GATA4 and GATA6 are Essential for Folliculogenesis, Ovulation, Corpus Luteum Function & Female Fertility

BY

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THESIS

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This thesis is dedicated to my family and friends. My thirst for knowledge, drive to succeed and perseverance through challenges is a result of the strong foundation of love, laughter and encouragement you have given me.

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LIST OF ABBREVIATIONS

3βHSD2	Type II 3β-Hydroxysteroid Dehydrogenase
ADAMTS1	A Disintegrin and Metalloproteinase with Thrombospondin Motifs 1
ADAMTS2	A Disintegrin and Metalloproteinase with Thrombospondin Motifs 2
AdCre	Adenoviral Cre-Recombinase
AMH	Anti-Müllerian Hormone
AMHR	Anti-Müllerian Hormone Receptor
Apoe	Apolipoprotein-E
cAMP	Cyclic Adenosine Monophosphate
CL	Corpus Luteum /Corpora Lutea
Comp	Cartilage Oligometric Protein
Cre	Causes Recombination
Cyp1b1	Cytochrome P450 Member, Family 1, Subfamily B, Polypeptide 1
Cyp11a1	Cholesterol Side Chain Cleavage Enzyme
Cyp17a1	17α-Hydroxylase/17,20 Lyase/17,20 Desmolase
Cyp19a1	Aromatase
dbcAMP	Dibutyryl Cyclic Adenosine Monophosphate
Depdc6	DEP Domain-Containing Protein 6
eCG	Equine Chorionic Gonadotropin
ECM	Extracellular Matrix
ERK1/2	Extracellular Signal Regulated Kinases 1/2
Fdx1	Ferrodoxin 1
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor

LIST OF ABBREVIATIONS (continued)

$G4^{gcko}$	GATA4 Granulosa Cell Conditional Knockout
$G4^{prko}$	GATA4 Progesterone Targeted Tissue Knockout
$G4/6^{gcko}$	GATA4/6 Granulosa Cell Conditional Knockout
$G4/6^{prko}$	GATA4/6 Progesterone Targeted Tissue Knockout
$G6^{gcko}$	GATA6 Granulosa Cell Conditional Knockout
$G6^{prko}$	GATA6 Progesterone Targeted Tissue Knockout
GCs	Granulosa Cells
GDF9	Growth Differentiation Factor-9
GnRH	Gonadotropin Releasing Hormone
Grem1	Gremlin 1
Grem2	Gremlin 2
H&E	Hematoxylin and Eosin
hCG	Human Chorionic Gonadotropin
HDL	High Density Lipoprotein
HIPK2	Homeodomain-Interacting Protein Kinase 2
HPO axis	Hypothalamo-Pituitary-Ovarian Axis
HSD17B1	Hydroxysteroid (17-Beta) Dehydrogenase 1
IGF1	Insulin-Like Growth Factor 1
IGFBP2	Insulin-Like Growth Factor Binding Protein 2
IGFBP4	Insulin-Like Growth Factor Binding Protein 4
IGFBP5	Insulin -Like Growth Factor Binding Protein 5
Inhα	Inhibin Alpha
Inhβa	Inhibin, Beta A

LIST OF ABBREVIATIONS (continued)

Inhβb	Inhibin, Beta B
IHC	Immunohistochemistry
ko	Knockout
L19	Mouse Ribosomal Protein L19
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Receptor
LH	Luteinizing Hormone
Lhcgr	Luteinizing Hormone/ Chorionic Gonadotropin Receptor
MAPK	Mitogen Activated Protein Kinase
Map3k5	Mitogen Activated Protein Kinase Kinase Kinase 5
mTOR	Mammalian Target of Rapamycin
OVGP1	Oviduct-Specific Glycoprotein 1
Papp-a	Pregnancy Associated Plasma Protein A
Pax2	Paired Box Gene 2
PCNA	Proliferating Cell Nuclear Antigen
PI3K	Phosphatidylinositol-3-Kinase
PIK3IP1	Phosphatidylinositol-3-Kinase Interacting Protein 1
PKA	Protein Kinase A
Pla2g4a	Phospholipase A2, Group IVA (Cytosolic, Calcium-Dependent)
PlxnC1	Plexin C1
PR	Progesterone Receptor
Prlr	Prolactin receptor
Prkar2b	Protein Kinase, cAMP-Dependent, Regulatory, Type II, Beta

LIST OF ABBREVIATIONS (continued)

qPCR	Quantitative Real Time Polymerase Chain Reaction
Ras	Rat Sarcoma
sc	Subcutaneous
Sema5a	Semaphorin 5A
Sema7a	Semaphorin 7A
StAR	Steroidogenic Acute Regulatory Protein
Vcan	Versican
VEGF	Vascular Endothelial Growth Factor
WT	Wildtype
ZP3	Zona Pellucida Glycoprotein 3

SUMMARY

Of the six GATA family members, only GATA4 and GATA6 are expressed in the adult ovary, specifically in granulosa, theca, and luteal cells. Although much work has been done on GATA4 and GATA6 to determine gene interactions through the overexpression of these transcription factors, their specific roles are still unknown in the ovary. Of interest, a number of genes important for ovarian function contain the GATA binding motif, WGATAR, including aromatase (Cyp19a1), cholesterol side chain cleavage enzyme (Cyp11a1), steroidogenic acute regulatory protein (StAR) and inhibin alpha (Inhα). As GATA factors are highly expressed in the ovary and have been shown to regulate genes crucial for ovarian function, the aim of this project was to identify the specific roles of GATA factors in female fertility, specifically during folliculogenesis through corpus luteum formation and to provide insight into the gene targets of these factors.

Single GATA6 ($G6^{gcko}$), GATA4 ($G4^{gcko}$), and double GATA4/6 ($G4/6^{gcko}$) granulosa cell-specific knockout mice were generated to investigate the role of GATA transcription factors in ovarian function *in vivo*. No reproductive defects were found in $G6^{gcko}$ animals. $G4^{gcko}$ animals were subfertile as indicated by the reduced number of pups per litter and the release of significantly fewer oocytes at ovulation. In contrast, $G4/6^{gcko}$ females fail to ovulate and are infertile. Furthermore, $G4/6^{gcko}$ females had irregular estrous cycles, which correlate with the abnormal ovarian histology found in unstimulated adult $G4/6^{gcko}$ females showing lack of follicular development and increased follicular atresia. Moreover, treatment with exogenous gonadotropins did not rescue folliculogenesis or ovulation in $G4/6^{gcko}$ mice. In addition, ovary weight and estradiol levels were significantly reduced in $G4^{gcko}$ and $G4/6^{gcko}$ animals when compared with control and $G6^{gcko}$ mice. The expression of Cyp19a1, Cyp11a1, and luteinizing hormone/chorionic gonadotropin receptor (Lhcgr) was significantly lower in $G4^{gcko}$ and $G4/6^{gcko}$ mice when compared with control animals. Most prominently, follicle stimulating hormone receptor (FSHR) protein was undetectable in granulosa cells of $G4^{gcko}$ and $G4/6^{gcko}$. Accordingly, gel

shift and reporter assays revealed that GATA4 binds and stimulates the activity of the FSHR promoter. These results demonstrate that GATA4 and GATA6 are needed for normal ovarian function. Our data are consistent with a role for GATA4 in the regulation of the *FSHR* gene and provide a possible molecular mechanism to explain the fertility defects observed in animals with deficient GATA expression in the ovary.

Knockdown of the transcription factors GATA4 and GATA6 in granulosa cells (GCs) impairs folliculogenesis and induces infertility. To investigate the pathways and genes regulated by these factors, we performed microarray analyses on wildtype (WT) GCs or GCs lacking GATA4, GATA6 or GATA4/6 ($G4^{gcko}$, $G6^{gcko}$ and $G4/6^{gcko}$) after *in vivo* treatment with equine chorionic gonadotropin (eCG). GATA4 deletion affected a greater number of genes than GATA6, which correlates with the subfertility observed in $G4^{gcko}$ mice and the normal reproductive function found in $G6^{gcko}$ animals. An even greater number of genes were affected by the deletion of both factors. Moreover, the expression of FSHR, Lhcgr, inhibin α and β, versican, pregnancy-associated plasma protein A, and the regulatory unit 2b of protein kinase A, which are known to be crucial for ovarian function, was greatly affected in double GATA4 and GATA6 knockouts when compared with single GATA deficient animals. This suggests that GATA4 and GATA6 functionally compensate for each other in the regulation of key ovarian genes. Functional enrichment revealed that ovulation, growth, intracellular signaling, extracellular structure organization, gonadotropin and growth factor actions, and steroidogenesis were significantly regulated in $G4/6^{gcko}$ mice. The results of this analysis were confirmed using qPCR, IHC, and biological assays. Treatment of GCs with cAMP/IGF1, to bypass FSH and IGF1 signaling defects, revealed that most of the affected genes are direct targets of GATA4/6. The diversity of pathways affected by the knockdown of GATA underscores the important role of these factors in the regulation of GC function.

Lack of follicle development in the GATA4/6^{gcko} precluded studies to examine the role of GATA4 and GATA6 in luteal cells where GATA factors are also expressed. Therefore, it is not known if these factors are involved in the regulation of luteal function *in vivo*. Our last aim was to determine the effect of the knockdown of GATA4 and GATA6 at ovulation on corpus luteum function and fertility. To delete GATA4/6 in the corpus luteum, mice expressing Cre recombinase driven by the progesterone receptor (PR) promoter, which is highly upregulated in the granulosa cells of the preovulatory follicle, were crossed with mice containing single or combined floxed alleles for GATA4 and GATA6 (G4^{prko}, G6^{prko}, G4/6^{prko}). G4/6^{prko} females produced no pups while both the G4^{prko} and G6^{prko} animals had a reduced number of pups per litter. G4/6^{prko} animals cycled normally and had normal hormone-induced ovulation rates. However, plasma progesterone levels and the ovarian expression of Cyp11a1 and StAR, both essential for progesterone synthesis, were significantly low in G4/6^{prko} mice treated with eCG/hCG (96 hs) when compared with control animals. Ovarian histology demonstrated that corpora lutea were present in the G4/6^{prko} animals treated with eCG/hCG (96 hs). Progesterone was administered to the double knockouts animals from day 1.5 to day 8.5 of pregnancy to determine if exogenous progesterone rescues pregnancy. This treatment rescued implantation in one out of two animals. In addition, structural abnormalities in the oviducts of G4/6^{prko} mice suggest the presence of luminal epithelium hypertrophy and tubular occlusion. Additionally, oviductal and uterine genes were significantly altered in the double knockout. Although, additional studies are needed to examine the effects of the deletion of GATA4 and GATA6 in other PR expressing tissues, such as the oviduct and uterus, these findings provide new insights into the roles these transcription factors have in female reproduction and demonstrate that they are not only crucial for the progression of folliculogenesis but also necessary for luteal cell progesterone production.

I. BACKGROUND

A. Female Reproductive System

1. Hypothalamo-Pituitary-Ovarian Axis

The hypothalamo-pituitary-ovarian (HPO) axis controls ovarian function and female fertility. Ovarian function relies on the tight regulation of hormones produced and secreted by the hypothalamus, the pituitary and the ovaries, themselves (1,2). The hypothalamus signals the pituitary through the pulsatile release of gonadotropin releasing hormone (GnRH). GnRH in turn acts on the pituitary gonadotrophs to stimulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) (3,4). FSH and LH target the ovary where they regulate the growth, differentiation and function of follicles. Follicles respond to gonadotrophins, in part by increasing the production and release of the steroid hormones, estradiol and progesterone. Estradiol and progesterone, in turn, feedback to the hypothalamus, as well as the pituitary, and regulate GnRH and gonadotrophin release. Release of LH and FSH from the pituitary is suppressed through inhibition of GnRH when estradiol is at low circulating levels during estrus. In contrast, high levels of estradiol at proestrus increase FSH and LH released, which promotes the LH surge seen at ovulation. The inhibition of LH and FSH released seen with low estradiol levels is enhanced by high levels of progesterone, in addition to blocking the positive feedback actions of estradiol on the pituitary (2,4).

Steroid hormones are not the only regulators of pituitary function. Inhibins and activins produced in the ovary can also regulate FSH within the HPO axis. Inhibins consist of the inhibin α subunit heterodimerized to either the inhibin βa or inhibin βb subunit. Activins are composed of the inhibin βa and inhibin βb subunits, heterodimerized to one another or from homodimerized beta subunits. Inhibins can act to suppress the release of FSH from the pituitary whereas activins promote the secretion of FSH from the pituitary (5,6). In vitro culture of human luteinized granulosa cells has also shown that FSH and LH can stimulate the expression of the inhibin α and inhibin βa subunits. Activins and insulin-like growth factor one also stimulate inhibin secretion (7).

LH and FSH are not the only gonadotropins released from the pituitary to affect ovarian function. Prolactin is also produced in the anterior pituitary and its secretion is regulated by the hypothalamus as well as itself. Dopamine from the brain is the predominant downregulator of prolactin secretion. Prolactin is essential for corpora lutea formation in the ovary as knockout mice of the prolactin receptor result in infertility as the corpora lutea undergo regression in pregnant animals at a faster rate than control animals (8,9). This is a result of the corpora lutea being the main contributor of progesterone production, which maintains implantation and pregnancy (10).

Normal ovarian function can be disrupted through impaired production, secretion or action of the hormones from the hypophyseal portal system (1,2,4). Mice lacking FSHR are infertile due to a lack of ovulation, loss of estradiol and progesterone production, increased testosterone production, atrophy of the uterus and reduced ovarian size (11-13). Similarly, LH receptor (Lhcgr) mutant female mice are hypogonadal and have decreased serum levels of estradiol and progesterone as well as ovarian defects in folliculogenesis including degenerating antral follicles and lack of corpora lutea (14).

2. Ovary

The roles of the ovary include the storage of primordial follicles and the gradual release of mature oocytes through a process called folliculogenesis. Folliculogenesis drives the activation and growth of primordial follicles in order to release a mature oocyte from the ovary into the oviduct to allow fertilization by sperm to occur. After activation, follicles progress from this primordial stage through primary, preantral and antral/preovulatory stages (depicted in Figure 1). Throughout this process, granulosa cells proliferate and differentiate under the tight control of hypophyseal hormones and locally produced factors. Thus, during the course of folliculogenesis autocrine, paracrine, and endocrine factors regulate the follicle's progress (15).

Another role, as mentioned earlier, is the production of hormones, including estradiol, progesterone, and inhibin, which coordinates the regulation of the HPO axis and the proliferation and preparation of the uterus for implantation.

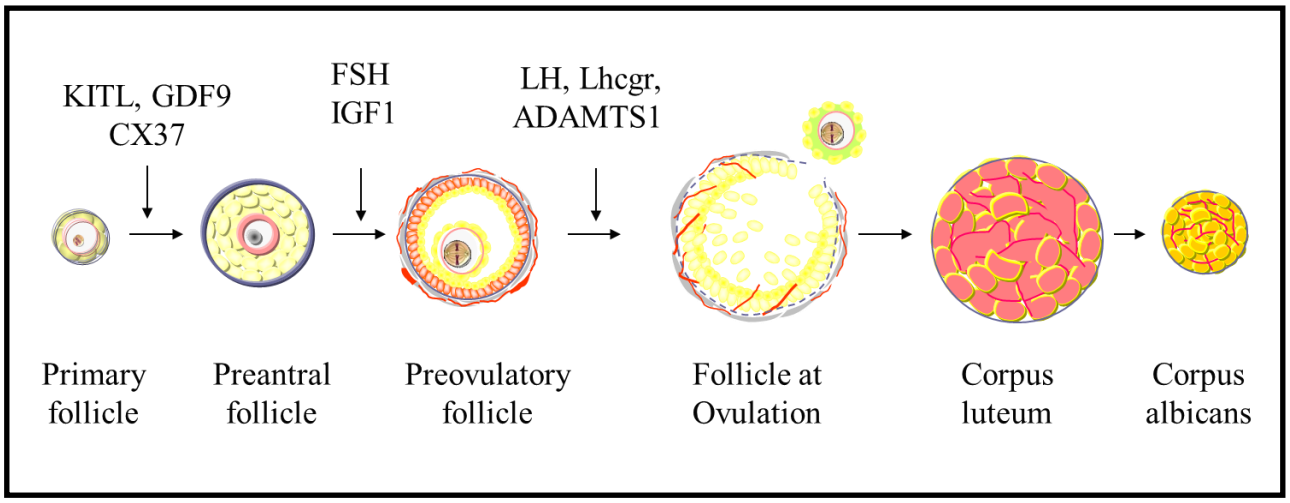


Figure 1. Folliculogenesis

During follicle development, a follicle will pass through the primary, preantral and antral/preovulatory stages until a mature oocyte is released through ovulation. The follicle then differentiates to form a corpus luteum, which ultimately undergoes luteolysis forming a *corpus albicans*. Different hormones and factors are crucial for the transition from one stage in folliculogenesis to the next. For example, KITL, GDF9 and CX37 are necessary for the transition from primary to preantral follicle. In contrast, FSH and IGF1 are both crucial for the transition between preantral to preovulatory follicles while PR, Lhcgr and ADAMTS1 are required for ovulation to occur. Mutation or deletion of the factors or hormones involved in folliculogenesis halts follicle development and impairs female fertility.

2.1. Folliculogenesis

2.1.1. Primordial and Primary Follicles

Primordial follicles consist of a single flat granulosa cell layer surrounding the oocyte, while the primary follicle consists of cuboidal granulosa cells surrounding the oocyte (15,16). The primordial follicles represent a finite reserve of oocytes that will remain in this inactive, primordial stage until their activation to the primary stage throughout the life of the female. How some primordial follicles become activated while others don't during a woman's lifetime is still unknown. However, it is believed that there are diffusible factors released from primordial follicles that inhibit the activation of neighboring primordial follicles (17). Factors that are crucial for primordial follicle formation include factor in the germ line alpha, gremlin 1, and bone morphogenetic protein 15 combined with growth differentiation factor 9 (GDF9) as knockout animals can result in the absence of primordial follicle formation or abnormal follicle formation (18-20). The activation of primordial follicles to form primary follicles is regulated by anti-mullerian hormone (21), forkhead box protein O3 (22), phosphatase and tensin homolog (23) and inhibin alpha (24) as deletion of each of these genes in mice results in an increased activation of the primordial follicle pool, leading to a decrease in the number of primordial follicles found in adulthood. Impaired progression of folliculogenesis after primordial follicle formation with subsequent increased depletion of follicles results in knockout mice for LIM-homeobox protein 8 (25), newborn ovary homeobox gene (26), and spermatogenesis and oogenesis specific basic helix-loop-helix protein 1 and 2 (27-29). Depletion of KIT ligand or proto-oncogene c-KIT can arrest follicle progression at the primary follicle stage and increases atresia (15,30-32).

2.1.2. Preantral Follicles

The preantral follicle is comprised of granulosa cells that have proliferated, forming several layers around the oocyte. Theca cells are recruited at this stage to surround the granulosa cells but the factors required for the differentiation of these cells are still unknown. At this stage, preantral follicle formation is gonadotropin independent and relies on paracrine and autocrine regulatory factors (15).

Genes necessary to form preantral follicles include GDF9, connexin 37 and neurotrophic tyrosine kinase receptor, type 2 (NTRK2). GDF9 null animals have reduced granulosa cell proliferation, do not recruit theca cells and halt their follicular growth at the primary stage (33,34). Connexin 37 gap junctions are necessary for oocyte-granulosa cell signaling as knockout mice only form preantral follicles and have premature luteinization (35). The neurotrophin, NTRK2 is necessary for the development of preantral follicles as null mice don't have follicle progression beyond the primary follicle stage (36).

2.1.3 Antral/Preovulatory Follicles

The antral/preovulatory follicle is distinguished by the presence of a fluid filled space within the follicle, called an antrum and the thinning of the follicle wall. The granulosa cells differentiate in which the cumulus granulosa cells maintain contact around the oocyte while the mural granulosa cells have no contact with the oocyte and are towards the exterior of the follicle. There is also additional proliferation of the granulosa and theca cells. Antral/preovulatory follicle development is gonadotropin dependent. FSH becomes a critical in preovulatory follicle formation and stimulates granulosa cell proliferation, prevents granulosa cell apoptosis, promotes estradiol production and Lhcgr expression. The classical signaling cascade of FSH acts through protein kinase A which results in the regulation of genes, such as aromatase, lhcgr and the inhibin subunits. Insulin-like growth factor 1 (IGF1) is another factor required for preovulatory follicle development. Signaling from IGF1 occurs through the protein kinase B pathway (which is also a non-classical signaling pathway for FSH) which influences granulosa cell proliferation via regulation of FSHR and aromatase expression (15). Thus, FSH and FSHR mutant mice have a halt in folliculogenesis at the preantral follicle stage (12,13,15,37) as do IGF1 knockout mice (38,39).

2.2. Ovulation

At ovulation, the oocyte is released from the mature follicle under the action of a surge of LH which results from the increased production of estradiol by the preovulatory follicles (40). LH acting through its receptor, lhcgr, regulates genes crucial for ovulation, including progesterone receptor (PR)

and cyclooxygenase 2 (COX2). Ovulation is characterized by the expansion of the cumulus oocyte complex which occurs through the production of an extracellular hyaluronan rich matrix (40) of which hyaluronan synthase 2 and COX2 are involved. These two factors contribute to the separation of the cumulus granulosa cells from each other and their progression outward into the follicle, away from the oocyte. The synthesis of a hyaluronan rich extracellular matrix is one means by which ovulation resembles the inflammation process. Versican (Vcan) and ADAMTS1 (a disintegrin-like and metalloproteinase with thrombospondin type I motif-1) are extracellular matrix proteins upregulated within the cumulus oocyte complex that are necessary for ovulation to occur (15,40,41). Mice lacking ADAMTS1 are subfertile having a higher number of apoptotic granulosa cells and trapped oocytes (41).

2.3. Corpus Luteum Formation

The corpus luteum (CL) forms as a result of both theca and granulosa cell differentiation where there is an invasion of theca cells and blood vessels into the ovulated follicle. LH induces the differentiation of follicular cells into luteal cells after ovulation and induces the exit of follicular cells from the cell cycle. Differentiation of the granulosa cells and theca cells into luteal cells as well as the vascularization of the CL involves multiple factors. In particular, loss of cyclin dependent kinase 2 (Cdk2) and the expression of Cdk inhibitors $p21^{cip1}$ and $p27^{kip1}$ lead to the termination of granulosa cell proliferation (10,42,43). Additionally, receptor activity changes during luteinization in which FSHRs disappear due to the upregulation of retinoic acid and LH while prolactin receptor (Prlr) and estrogen receptor alpha become highly expressed, which is needed to sustain corpus luteum function (10).

Other factors that contribute to the normal formation and function of the CL include, CATT/enhancer binding protein β (C/EBPβ), COX2 and PR, and early growth response protein 1. C/EBPβ knockout mice are able to ovulate but fail to form CL. In contrast COX2, PR and early growth response protein 1 knockout mice fail to ovulate but do form CL (10,44-46). Each of these genes are upregulated by the LH surge but the knockout animals fail to respond to exogenous gonadotrophins.

The structure of the CL differs from that of the follicle. The CL is highly vascularized as each luteal cell is in direct contact with capillaries. The development of these capillaries relies on the degradation of the extracellular matrix, endothelial cell proliferation, and existing capillary expansion. The vascularization of the CL is crucial as it supplies the CL with the cholesterol necessary to produce progesterone, and the means by which progesterone can affect other areas of the body. Vascular endothelial growth factor (VEGF) is required for the vascularization process as it is only expressed in the luteal cells of the ovary and lack of VEGF results in the absence of CL in the ovary (10).

The major function of the CL is to produce progesterone (10). Progesterone stimulates the growth of blood vessels that supply the lining of the endometrium and stimulates endometrial glands to secrete nutrients that nourish the early embryo. Thus, progesterone prepares the uterus to allow the fertilized egg to implant and helps to maintain the endometrium throughout pregnancy (8).

2.4. Steroidogenesis

One of the main roles of the ovary is to synthesize steroid hormones which include progestins, estrogens and androgens. Steroids are derived from cholesterol, which can be made *de novo*, through the hydrolysis of lipid stores, and derived from lipoproteins in circulation (10) including high and low density lipoproteins (HDLs and LDLs). The CL primarily utilizes HDLs, which are bound by scavenger receptor class B type I in mice (10,47,48). Once in the cells, cholesterol can be stored in lipid droplets or transported into the mitochondria by steroidogenic acute regulatory protein (StAR). In the mitochondria, cholesterol is converted into pregnenolone by action of the P450 side chain cleavage (Cyp11a1) enzyme. Pregnenolone is reduced into progesterone through type II 3β-hydroxysteroid dehydrogenase (3βHSD2) (10). Progesterone metabolism occurs through the enzyme 20-α-hydroxysteroid dehydrogenase, but during pregnancy, this enzyme is inhibited to maintain high levels of progesterone. The stimulation of progesterone production occurs through LH.

Progesterone is secreted mainly by luteal cells although it is also produced to a lesser degree in granulosa and theca cells of preovulatory follicles. (See Figure 2 for steroidogenesis in granulosa and

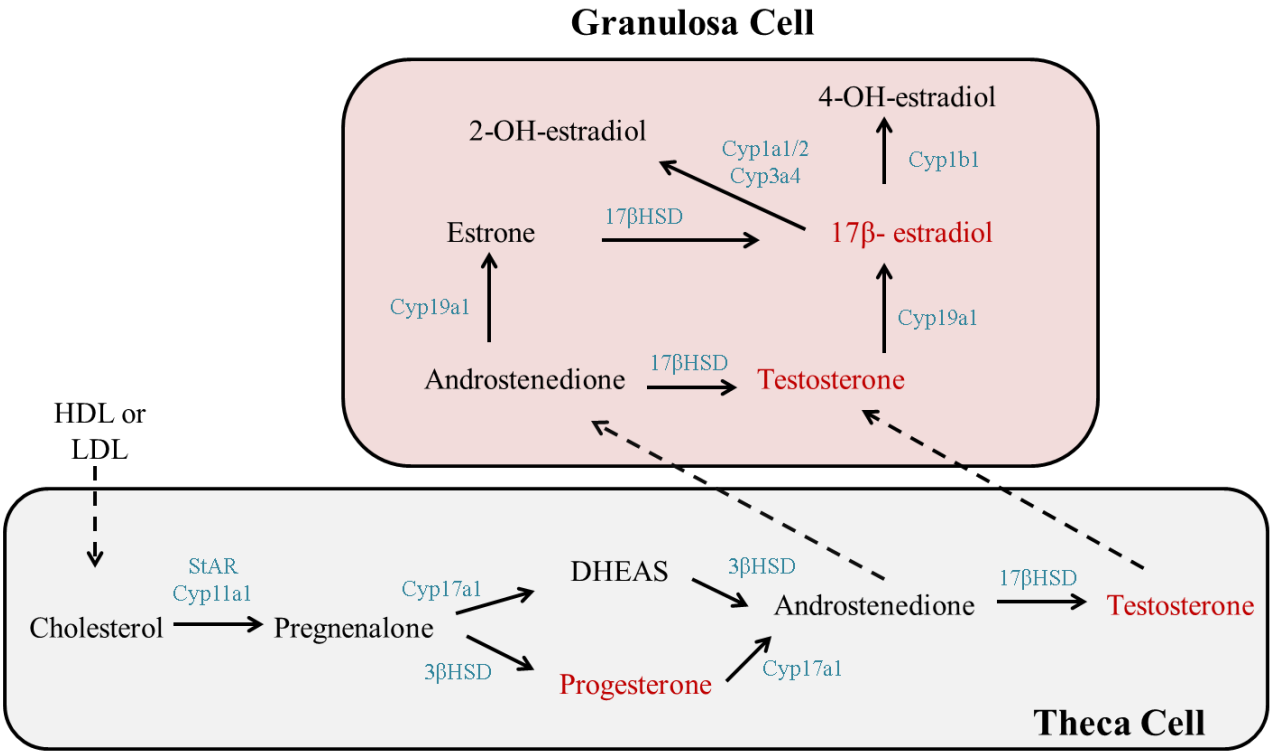


Figure 2. Steroidogenesis in Granulosa and Theca Cells

For steroidogenesis in theca cells, cholesterol derived from HDLs or LDLs is converted to pregnenalone through StAR and Cyp11a1. Pregnenalone can either be converted to progesterone by 3βHSD or converted to the androgen DHEAS. Progesterone can be converted to the androgen androsteinedione by Cyp17a1. DHEAS can also be converted to androsteinedione but by 3βHSD. Androsteinedione can then be converted to testosterone by 17βHSD. For steroidogenesis in granulosa cells, androstenedione and testosterone from the theca cells diffuse to the granulosa cells. Androsteinedione can be converted to testosterone by 17βHSD or estrone by Cyp19a1. Testosterone can be converted to 17β-estradiol by Cyp19a1. Estrone can be converted to 17β-estradiol as well, only by 17βHSD. 17β-estradiol can then be metabolized to 2-OH-estradiol by Cyp1a1/2 or Cyp3a4. 17β-estradiol can also be metabolized to 4-OH-estradiol by Cyp1b1. Steroidogenic enzymes are in blue. Progesterone, testosterone and estradiol, the main ovarian steroids are in red with all other steroids being in black. Dashed arrows indicate movement of steroids while solid arrows indicate synthesis/conversion by the enzyme(s) listed next to it.

theca cells.) One of the primary functions of the theca cells is to convert progestins to androgens by action of the Cyp17a1 (17α-hydroxylase) enzyme (49,50). Androgen production is in part regulated by the transforming growth factor family, which act to suppress androgen production through the SMAD 2/3 pathway. The bone morphogenetic proteins 4/6/7 also suppress Cyp17a1 activity, but through the SMAD 1/5/8 pathway (51). Androgen production (testosterone and androsteinedione) is necessary in the ovary as it acts as a substrate for the synthesis of estrogens (17β-estradiol and estrone) and synergizes with FSH to stimulate aromatase activity (49,52). Our lab has also found that androgens alone can increase the mRNA expression of Cyp19a1 and Cyp11a1 (53). In granulosa cells, androgens are converted into estrogens as they are the only cells that express the aromatase (Cyp19a1) enzyme (52). Regulation of aromatase occurs through its activation by FSH during preovulatory follicle formation and suppression by prolactin and LH during pregnancy (52). Additional regulation of estradiol occurs through its metabolism by cytochrome 450 1B1 (Cyp1b1) and cytochrome 450 1A1 (Cyp1a1). Infertility occurs with the dysregulation of ovarian steroid hormones (15). For example, mutant females lacking the aromatase enzyme are infertile, and follicles only progress to the antral stage, preventing ovulation. These animals also have high testosterone, LH and FSH levels and develop hemorrhagic follicles (54).

B. GATA Factors

Follicle selection and atresia are highly regulated pathways controlled by genes involved in differentiation, cell survival, steroidogenesis and apoptosis. Several genes included in these diverse processes contain within their regulatory elements the motif WGATAR (55-57). This motif is recognized by, and gives name to, a small family of transcription factors identified as GATA (55,58,59). GATA factors have been shown to regulate the expression of genes involved in follicle growth and steroid synthesis (55).

1. GATA Expression

There are six members in the GATA family of transcription factors but GATA4 and GATA6 are the only isoforms expressed in the ovaries of adult animals (60-62). GATA4 is expressed in granulosa cells as the primordial follicle matures to the primary stage (62). GATA4 is highly expressed within the granulosa and minimally expressed in the theca cells of the ovary. The expression of GATA4 gradually increases as the follicle mature. GATA4 has also been shown to be expressed in the luteal cells of the ovary (61,63) and in the epithelial cells of the oviduct and uterus as well as the stromal cells of the uterus (Stocco unpublished; Figure 3).

GATA6 has been localized in the gut, heart, liver and lung (64). Like GATA4, GATA6 is highly expressed within the ovary but it is also found in oocytes in addition to granulosa, theca and luteal cells (61,63). Our lab has also detected GATA6 in the epithelial cells of the oviduct and uterus as well as the stromal cells of the uterus (Stocco unpublished; Figure 3).

2. GATA Factors Structure

GATA4 and GATA6 contain within their structures two zinc finger domains that recognize the WGATAR binding motif (59). Specifically, the C-terminal finger is needed for site specific recognition to the GATA motif, whereas the N-terminal finger contributes to specificity and stability of GATA binding (65,66). The GATA structure also contains three transactivation domains as well as a nuclear localization signal (67,68).

There is high homology between the structures of the GATA factors, especially in their zinc finger binding domains (55). Shown in Figure 4, GATA4 and GATA6 protein structures are 85% homologous at their DNA binding domains (zinc finger domains) while only having 25% homology in the N terminal activation domains and 39% homology in the C terminal activation domain (57). The transcriptional activation domains allow GATA factors to bind to other proteins as well as undergo posttranslational modifications (55). The similarities between the GATA factors suggest that they can

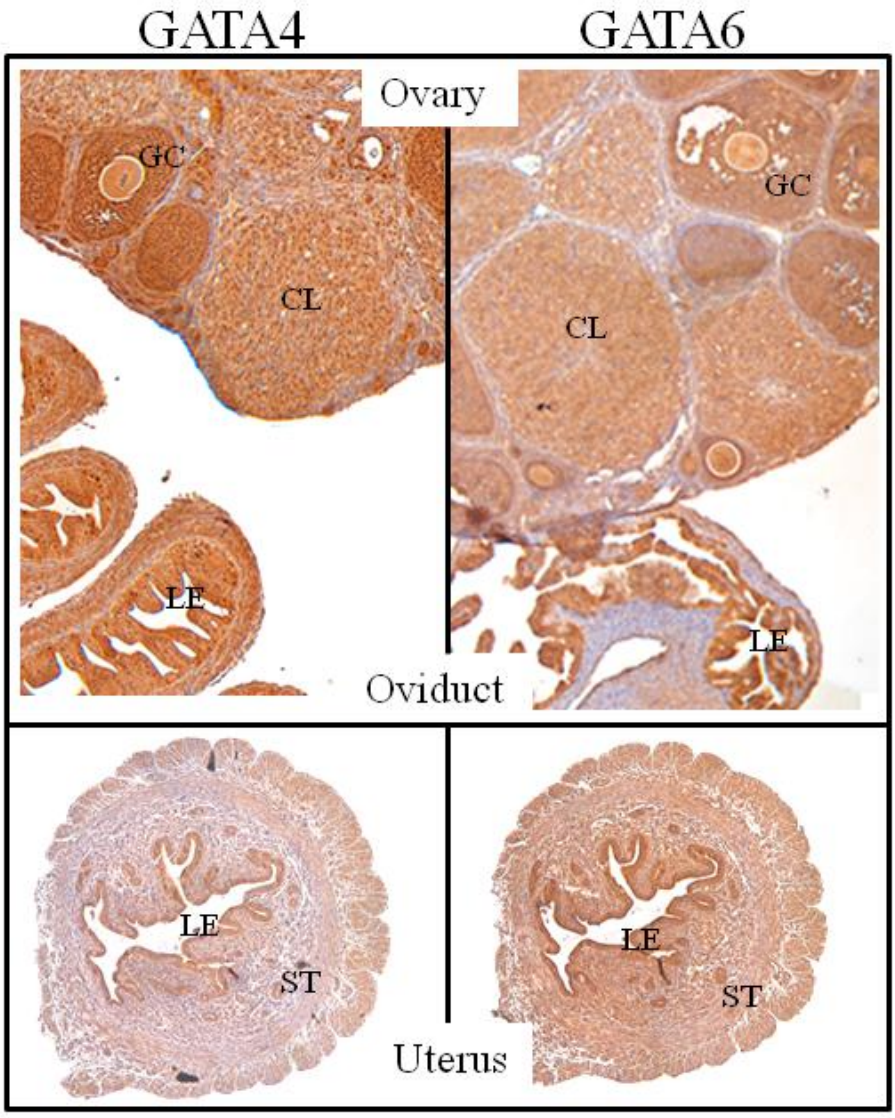


Figure 3: GATA Expression in Reproductive Tissues

GATA4 (*left*) and GATA6 (*right*) are highly expressed in the granulosa cells (GC) and corpora lutea (CL) of the ovary. They are also highly expressed in the luminal epithelium (LE) of the oviduct and uterus as well as being expressed in the stroma (ST) of the uterus.

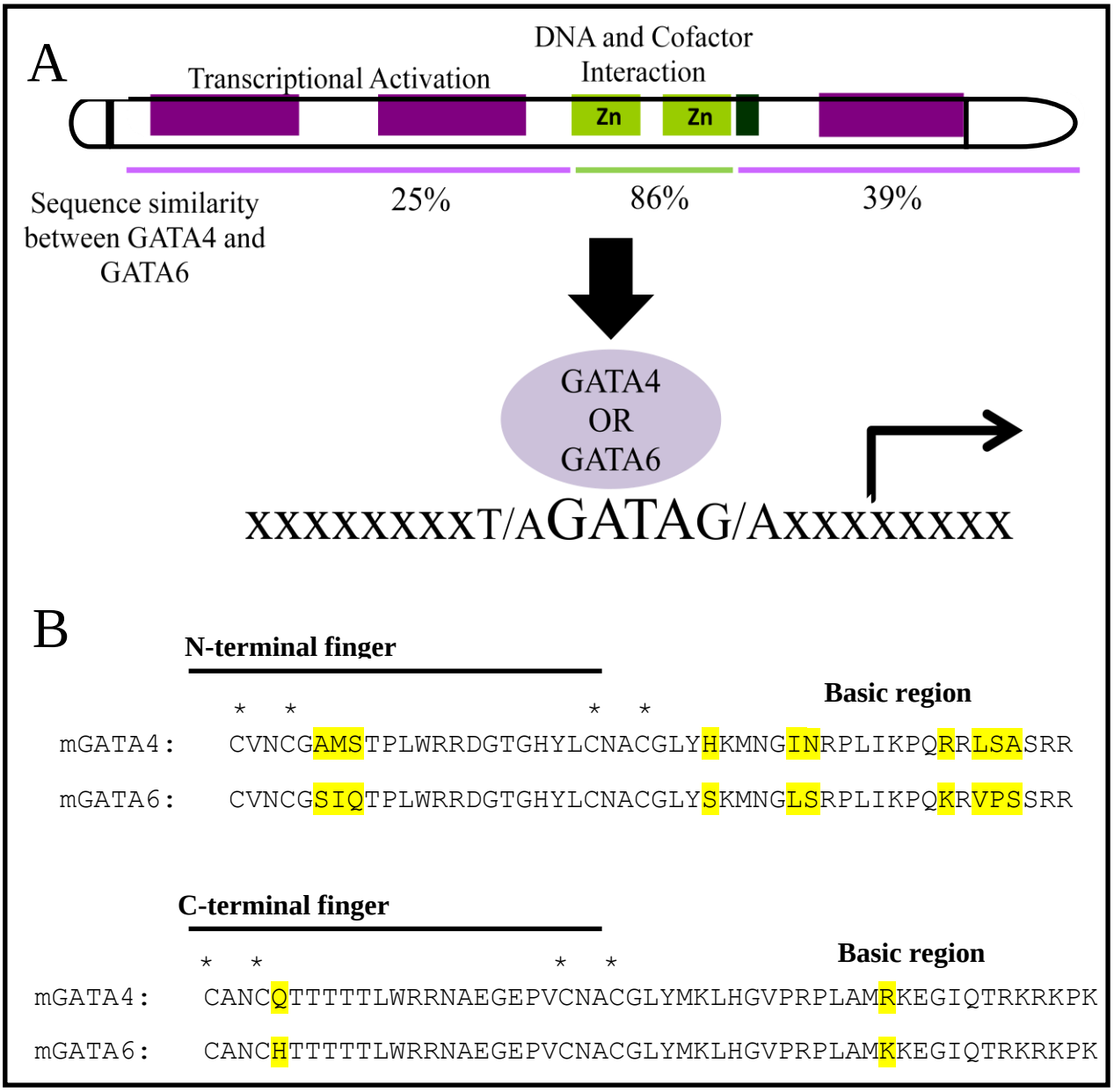


Figure 4. Homology between GATA4 and GATA6 Factors Structure.

A) GATA4 and GATA6 both contain two transcriptional activation domains located at the N terminus and one at the C terminus (*purple*). There are two zinc finger domains (Zn, *light green*) and a nuclear localization signal (*dark green*) towards the C terminus. GATA4 and GATA6 only have 25% and 39% homology at their N and C termini, respectively, but have 86% homology at their DNA binding (zinc finger) domains which allows them to bind the same W-GATA-R motif on gene promoters. **B**) Sequence of the mouse GATA4 and GATA6 zinc finger domains. Asterisk denote the cysteine residues of the zinc fingers. Yellow highlight denotes sequence differences between the two factors.

bind to the same genes to affect function, but their differences suggest that they may regulate genes differently.

3. GATA and Ovarian Gene Regulation

GATA4 and GATA6 bind and enhance the promoter activity of many genes that are important for normal ovarian function such as aromatase (Cyp19a1), Steroidogenic acute regulatory protein (StAR), and Inhibin-α. Similar findings have been found with GATA6 (reviewed in reference (56).

In vitro studies: Overexpression of GATA4 and GATA6 stimulates StAR expression and activates the promoter of Cyp19a1 and Inhibin-α (69,70). Transfection of granulosa cells with a reporter construct carrying a mutation of the GATA binding site on the Cyp19a1 promoter results in decreased stimulation of the promoter by FSH. Accordingly, overexpression of GATA4 synergizes with FSH in the stimulation of Cyp19a1 expression and promoter activity (71). GATA4 knockdown in luteinized porcine granulosa cells doubled cAMP (cyclic adenosine monophosphate) induction of StAR mRNA while the combination of GATA4 and GATA6 knockdown prevented cAMP induction of StAR. However, GATA6 alone was unable to alter StAR. Additionally, use of siRNA for both GATA factors resulted in an increase in the basal expression of Cyp11a1 but was unable to affect Cyp11a1 induction by cAMP. It was also found that GATA4 knockdown is able to reduce 3βHSD mRNA levels (72).

***In vivo studies*:** *In vivo* studies to determine the reproductive role of GATA factors have not been conducted because of the embryonic lethality of GATA4 or GATA6 null mice (58,73). Interestingly, FSHR-null mice, which have follicular arrest at the preantral stage, showed decreased GATA4 protein expression compared to controls (74).

4. Regulation of GATA4 and GATA6 Expression and Activity

GATA4 protein and mRNA levels increase with FSH stimulation (61,71). Phosphorylation and protein-protein interactions play a critical role in the regulation of the transcriptional activation of GATA factors (69). We have demonstrated that GATA4 phosphorylation at serine 105 by FSH is crucial for the induction of Cyp19a1 promoter activity and expression (71). ERK1/2 and PKA seem to be

involved in the activation of GATA4 by phosphorylating serine105 and serine261 respectively (71,75). Activation of GATA factors can also be modulated by sumoylation and acetylation in addition to phosphorylation (reviewed, (55); Stocco unpublished). These modifications may alter GATA nuclear localization, DNA binding, protein stability, and cofactor recruitment (reviewed, (55). However, their roles have not been clearly established.

In addition, interaction of GATA with other transcription factors and co-regulators affects its transcriptional activity. For instance, Friend of GATA factors 1 and 2 have been shown to interact with and repress or promote GATA activity depending on the tissue (76-78). Liver receptor homolog 1 and steroidogenic factor 1 (two nuclear receptors) have also been shown to interact with both GATA factors in synergy to activate the human 3βHSD2 promoter (79). The mechanisms involved in the regulation of GATA activity within the ovary remain to be determined.

It also appears that GATA4 and GATA6 could regulate one another. In GATA4 null mice GATA6 is upregulated while GATA6 null mice show a downregulation of GATA4. Thus, GATA4 appears to negatively regulate GATA6 expression while GATA6 positively regulates GATA4 expression. However, the mechanism behind this regulation is still unknown (57). Additionally, the regulation seen between the GATA factors could be concentration dependent and/or tissue specific.

C. Statement of Hypothesis and Aims

As described above, follicle development is crucial for normal female fertility. Follicle development requires the expression and repression of genes that are controlled by a specific combination of ovarian transcription factors. We hypothesize that GATA4 and GATA6 regulate granulosa cell differentiation, proliferation, and apoptosis and therefore, they are crucial for the normal progression of folliculogenesis.

The overall aim of this project was to determine, *in vivo*, the consequences of the specific inactivation of the GATA4 and/or GATA6 genes on ovarian function and female fertility. In our first

aim, we generated animals with conditional GATA4 and/or GATA6 knockdown in granulosa cells prior to preovulatory follicle formation, and then characterized and assessed the reproductive function of these mice. In our second aim, a microarray was performed to determine genome-wide GATA4 and GATA6 target genes in granulosa cells *in vivo* to uncover the genes as well as potential functional pathways that GATA factors regulate within the ovary. This experiment also provided information on unique as well common genes regulated by GATA4 and GATA6. Lastly, our third aim was to generate animals with conditional GATA4 and GATA6 knockdown in granulosa cells after preovulatory follicle formation at the time of ovulation and then characterize and assess the reproductive function of these mice. Completion of these aims unraveled novel functions of GATA factors in folliculogenesis.

II. MATERIALS AND METHODS

A. Materials and Reagents

DMEM/F12 medium was purchased from Invitrogen (Carlsbad, CA). Equine chorionic gonadotropin (eCG), estradiol, human chorionic gonadotropin (hCG), and all buffer components were purchased from Sigma (St. Louis, MO) unless otherwise stated. The antibodies for GATA4 (sc9053 and sc1237) and GATA6 (sc9055 and AF1700) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and R&D systems (Minneapolis, MN). β-actin (ab8227) and Lamin B1 (ab16048) were purchased from Abcam (Cambridge, MA). FSHR (S0522) and proliferating cell nuclear antigen (PCNA) (#2714-1) were purchased from Epitomics (Burlingame, CA). Cleaved caspase 3 (#9661) was purchased from Cell Signaling Technology (Danvers, MA). Versican (Vcan) (AB1033) was purchased from Millipore, Billerica, MA. Vascular endothelial growth factor (VEGF) (sc152) was purchased from Santa Cruz and paired box 2 (Pax2) (71-6000) was purchased from Invitrogen. Secondary antibodies (6721 and 305-035-045) were purchased from Abcam and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

B. Experimental Animal Protocols

1. Experimental Animals

Animals were treated in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all protocols were approved by the University of Illinois at Chicago Animal Care Committee (See Appendix B). GATA6F/F, Zp3-Cre and AMHR-Cre animals were obtained from The Jackson Laboratory (Bar Harbor, ME). GATA4F/F animals were kindly provided by Dr. William Pu (Children's Hospital, Boston, MA). The generation of Cyp19-Cre has been previously described (80,81) and were kindly provided by Dr. Joanne Richards (Baylor College of Medicine, Houston, TX). The generation of the PR-Cre has been previously described (82) and were kindly provided by Dr. Francesco DeMayo (Baylor College of Medicine, Houston, TX).

2. Genotyping

DNA was isolated from tail snips from immature mice (~d16). Tail snips were solubilized in buffer containing 1% SDS, 0.3M NaAc, 1mM EDTA and 10mM Tris (pH 7) and 0.02mg proteinase k (Invitrogen). Tails were incubated at 60°C until fully digested. Tubes were cooled and salt solution containing 4.21M NaCl, 0.63M KCl, and 10mM Tris-HCL (pH 8) was added. Tails were incubated for 10 min at 4°C and then spun down 10 min at 13,000 rpm at 4°C. Supernatant was transferred to a new tube and DNA was precipitated with two volumes of 100% ethanol for 30 min at -20°C. Samples were spun down 10 min at 13,000 rpm at 4°C and the resulting pellet was washed with 80% ethanol. Samples were spun down for 10 min at 13,000 rpm at 4°C and pellet was air dried. Pellet was then resuspended in 50µl 1x TE buffer and heated 10 min at 60°C.

Wildtype, floxed, Cre and null alleles were determined by PCR utilizing the primers listed in Table I. PCR protocols are the following: GATA4, denature at 94°C for 2 min followed by 35 repeats of 94°C at 10 sec, 50°C at 30 sec and 72°C at 1 min, following the repeats samples had a final extension at 72°C for 5 min. $GATA4^{ko}$, GATA6 and $GATA6^{ko}$, denature at 94°C for 3min followed by 35 repeats of 94°C for 10 sec, 58°C for 1 min, and 72 °C for 1 min, after the repeats the PCR was completed with a final extension of 72°C for 5 min. Cyp19-Cre, denature at 94°C for 2 min followed by 30 repeats of 92°C for 20 sec, 60°C for 20 sec and 73°C for 45 sec, after the repeats the PCR was completed with a final extension for 2 min at 72°C. Zp3-Cre, denature at 94°C for 3 min followed by 35 repeats at 94°C for 15 sec, 53°C at 30 sec and 72°C at 1 min, after the repeats the PCR was completed with a final extension at 72°C for 5 min. PR-Cre, denature at 94°C for 5 min followed by 35 repeats of 94°C at 1 min with 58°C for 1 min 45 sec and 72°C for 2 min completed with a final extension of 72°C for 10 min.

Loading dye containing sybr was added to the PCR products and they were run on agarose gels. Bands were detected by UV light. The presence or absence of bands as well as the molecular weight dictated the genotype of the mouse. The molecular weight of the PCR product for GATA4 wildtype is

Gene	Forward	Reverse
GATA4	GGTGGTTTCATTTGCTGTGTGGAAG	CCTTGCTTTCTGCCTGCTACACAC
$GATA4^{ko}$	TGTCATTCTTCGCTGGAGCCGC	TCCTTTGCTGATCTCCTC
GATA6	GTGGTTGTAAGGCGGTTTGT	ACGCGAGCTCCAGAAAAAGT
$GATA6^{ko}$	AGTCTCCCTGTCATTCTTCCTTGCTC	TGATCAAACCTGGGTCTACACTCCTA
Zp3-Cre	GTGAAACAGCATTGCTGTCACTT	GGACATGTTCAGGGATCGCCAGGCG
Cyp19-Cre	ACTTGGTCAAAGTCAGTGCG	TACAGCACCCTCTGAAGCAA
PR-Cre	F1:ATGTTTAGCTGGCCCAAATG F2:CCCAAAGAGACACCAGGAAG	TATACCGATCTCCCTGGACG

TABLE I. GENOTYPING PRIMERS

Forward and reverse primers used during PCR for genotyping experimental mice. Primers are listed 5' to 3'

240bp; floxed GATA4 is 280bp and GATA4^{ko} is 330bp. The molecular weight of the PCR product for GATA6 wildtype is 159bp; floxed GATA6 is 250bp and GATA6^{ko} is 180bp. The molecular weight of the PCR product for Cyp19-Cre is 500bp, Zp3-Cre is 250bp and PR-Cre is ~600bp with its WT band at ~300bp. Presence of a band in the ko, Zp3-Cre and Cyp19-Cre indicate the animal contains the allele. Example genotyping results are shown in Figure 5.

3. Estrous Cycling

Vaginal smears using 20µl of PBS were collected daily from adult females (~d55) over the course of 12 days. The vaginal smears were evaluated microscopically to identify the stage of the estrous cycle (nucleated cell- proestrus, cornified cells-estrus, cornified cells and leukocytes-metaestrus, leukocytes-diestrus).

4. Superovulation

Adult females (~d90) were injected sc with 7.5 IU of eCG followed by 7.5 IU of hCG 48 hs after to promote ovulation. Animals were killed 17 hs after hCG and oocytes from each oviduct were collected, counted and deemed viable or dead. Oocytes were deemed dead if they had darkened or irregular shaped nuclei.

5. Ovarian Weight Determination

After the surgical dissection of the ovaries, the surrounding fat was removed and the wet weight was determined on an analytical balance.

6. Pregnancy Assessment at Day 9

Control and G4/6^{prko} females (> 42 days old) were paired with proven males. The day the vaginal plug was detected was considered day 1 of pregnancy. Upon detection of a plug, G4/6^{prko} animals were injected with 3mg/ml progesterone sc daily from day 1 until day 9, pregnant control animals were injected with an equivalent amount of sesame oil for the same length of time. Tissues were then harvested and fixed in formalin.

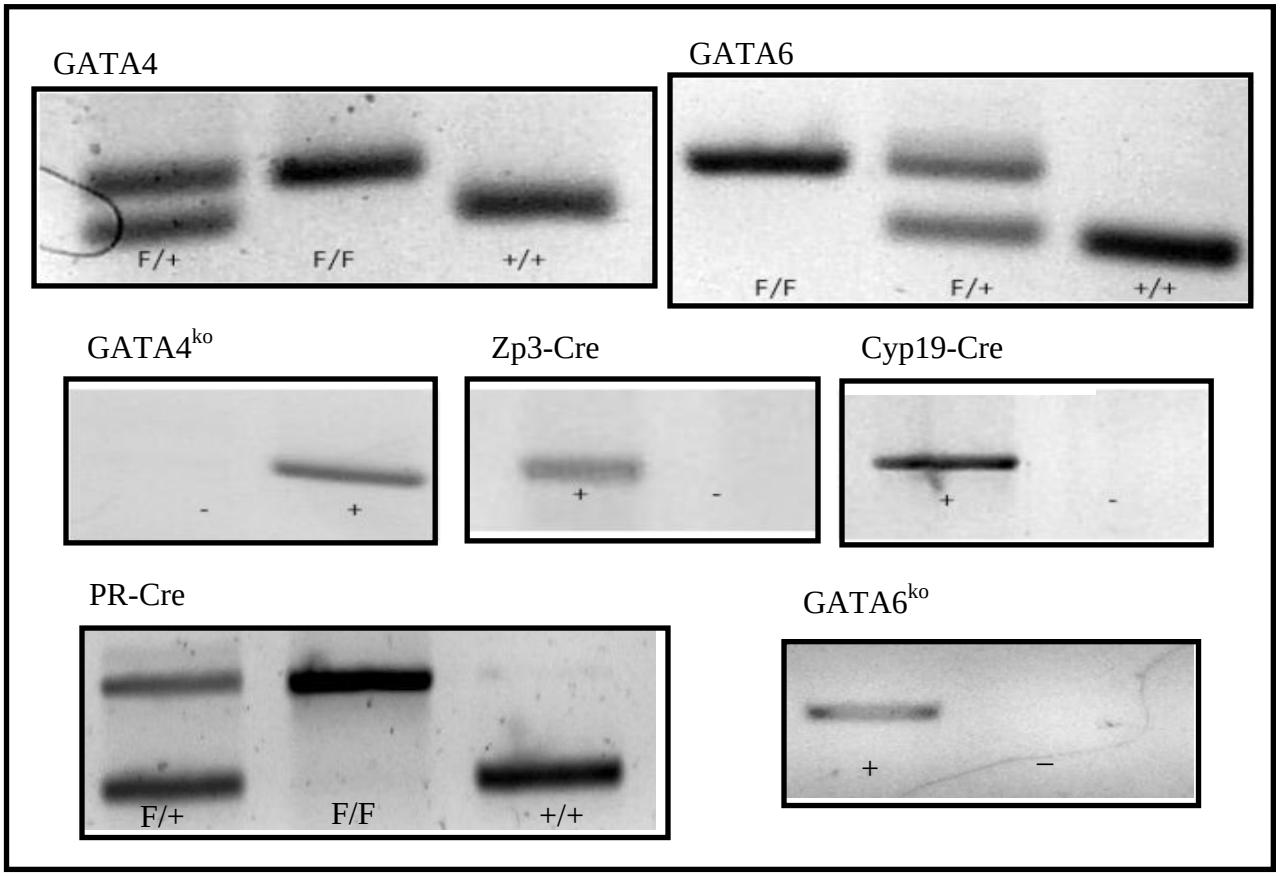


Figure 5. Mice Genotyping Results

Molecular weight (MW) of the PCR product for GATA4 WT is 240bp; floxed GATA4 is 280bp and GATA4 ^{ko} is 330bp. MW of GATA6 WT is 159bp; floxed GATA6 is 250bp and GATA6^{ko} is 180bp. MW of Cyp19-Cre is 500bp, Zp3-Cre is 250bp, PR-Cre is ~600bp and its WT band is ~300bp. Presence of a band in the GATA4^{ko} or GATA6^{ko}, Zp3-Cre and Cyp19-Cre indicate the animal contains the allele.

C. Hormone Assessments

Truncal blood was collected using lithium heparin tubes (Sarstedt, Newton, NC), and plasma was isolated following manufacturer's instructions. In chapter III and IV, estradiol and progesterone plasma levels were determined by using estradiol and progesterone EIA kits (Cayman Chemical Co., Ann Arbor, MI), respectively. Estradiol concentration was determined in undiluted plasma samples whereas progesterone plasma levels were used at a 1:10 dilution. FSH and LH plasma levels were determined by The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. In chapter V, the progesterone EIA kit used was from DRG Diagnostics, Marburg, Germany. Progesterone plasma levels were used at a 1:5 dilution.

D. Cell Isolation and Culture

1. Primary Granulosa Cell Isolation

Wildtype (WT), $G4^{gcko}$, $G6^{gcko}$, and $G4/6^{gcko}$ immature (D23) female animals were injected sc with 7.5 IU eCG, and ovaries were harvested after 48 hs. Animals were anesthetized with isoflurane and then cervically dislocated. Ovaries were dissected and placed in DMEM/F12. Granulosa cells (GCs) were isolated from preovulatory follicles in WT, $G4^{gcko}$, $G6^{gcko}$, and $G4/6^{gcko}$ animals or from large preantral follicles in $G4/6^{gcko}$ mice, which do not form preovulatory follicles. Cells were filtered through 40 μM mesh to partially eliminate cumulus oocyte complexes. Cells were resuspended in either TRIzol reagent for RNA isolation or radioimmune precipitation assay buffer for protein isolation (see below).

2. Granulosa Cell Culture

Undifferentiated GCs were isolated from preantral follicles from D23 mice treated for 3 days with 1mg/ml estradiol. Cells were cultured as previously described (83). Cells were transfected with adenoviral Cre-recombinase (adCre; University of Iowa Gene Vector Transfer Core) at a MOI of 10.

2.1. MTT Assay

Undifferentiated GCs were treated with or without 100ng/ml FSH for 48 hs. Then 50µl of 50 mg/ml MTT dissolved in PBS was added to each culture well. Plates were incubated for 2 hs at 37°C. After incubation, medium was removed and 500µl of DMSO was added. Plates were shaken for 5 min at room temperature and the absorbance was read at 560nm.

2.2 dbcAMP Treatment

For RNA expression, cells were treated with adCre for 24 hs prior to treatments with 1mM dbcAMP (Sigma), 50ng/mL IGF1 (Sigma) or both. Cells were cultured for 48 hs after treatments and then harvested for RNA isolation.

3. Oocyte Culture

For oocyte culture, oocytes were collected from the large antral follicles. Oocytes were counted and then assessed for either being surrounded by cumulus granulosa cells or if they were denuded (surrounded by one layer or less of cells). Oocytes surrounded by cumulus cells were cultured in TCM 199 medium containing 10% FBS, 1µg/mL estradiol, 24.2mg/ml sodium pyruvate, 10ng/mL EGF (Irvine Scientific, Santa Ana, CA) and 10IU/mL PMSG. Oocytes were pooled for animals with the same genotype and allowed to mature overnight in media under oil. The oocytes were denuded in the morning with hyaluronidase and stage of oocyte maturation was determined. Oocytes at germinal vesicle stage (GV) still had a germinal vesicle surrounding the nucleolus. Oocytes at the GV/meiosis II (GV/MII) stage did not contain a germinal vesicle and did not have a distinct nucleolus. Oocytes at the meiosis II (MII) stage had a polar body present.

4. PR-Cre Tissue Isolation

Immature control and G4/6^{prko} mice were treated with 7.5 IU eCG for 48 hs followed by 7.5 IU hCG for 96 hs to promote corpora lutea formation. Animals were anesthetized with isoflurane and then cervically dislocated. Whole ovaries, oviducts and uteri were dissected from these animals and either fixed for immunohistochemistry or homogenized for RNA isolation.

5. Luteal Cell Culture

Luteal cells were isolated from immature female mice treated with 7.5 IU eCG for 48 hs followed by 7.5 IU hCG for 7 hs and cultured as previously described (83). Cells were transfected with adCre at a MOI of 10 and were cultured for 72 hs and then harvested for RNA isolation.

E. RNA Isolation and Quantitative Real-time PCR Analysis

Total RNA from primary mouse granulosa and luteal cells was isolated using TRIzol-Reagent (Invitrogen) following the manufacturer's instructions. Additionally, total RNA from whole ovaries, oviducts and uteri, which were initially homogenized, was isolated using TRIzol. For mRNA analysis by real time PCR, 0.5-1μg of the total RNA was reverse transcribed at 42°C using Moloney murine leukemia virus reverse transcriptase and later diluted to a final volume of 100μl. To generate standard curves, the cDNA of our genes of interest was cloned into the pCR2.1 vector (Invitrogen), sequenced, and excised by restriction enzyme. Purified cDNA was diluted to concentrations ranging from 10×10^2 to 6×10^6 copies/μl. Aliquots (5 μl) of standard cDNA or sample cDNA were combined with SYBR Green I (Bio-Rad Laboratories, Inc., Hercules, CA) and primers specific for the gene of interest. Only intron-spanning primers were used for PCR amplification. The list of all primers used in this chapter as well as subsequent chapters is listed in Table II.

Real-time quantification of the PCR product in each cycle was carried out in an iQcycler Real Time PCR machine (Bio-Rad) with the following cycling conditions: preincubation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 10 sec, and extension at 72°C for 40 sec. The melting peak of each sample was routinely determined by melting curve analysis to ascertain that only the expected products had been generated. The identity and size of all PCR products were confirmed by sequencing and gel analysis. The minimal number of cycles sufficient to produce detectable levels of threshold fluorescence (Ct) was calculated using MyiQ software. For each gene of interest the number of mRNA molecules was calculated using a standard curve and expressed as copies

Gene	Forward	Reverse
ADAMTS1	AAGCTCACCTGTGAAGCCAAAGG	GTTTCACTTCAATGTTGGTGGCTCC
ADAMTS2	TGTGTTGCTGAGGTTTCG	CTGATCCTGACTCACCTATCC
AMH	AGTGAGGGTCTCTAGGAAGG	CATCTTAACCCTTCAACCAA
Comp	CAGACCATGAACAGTGACC	TCTGTTTCCACATGACTACG
Cyp1b1	ATCAAAGTCCTCTGGGTTAG	CTGCTCATCCTCTTTACCA
Cyp11a1	GATGTTCCACACCAGTGTCCC	AGGGTACTGGCTGAAGTCTCGC
Cyp19a1	ATTGCAGCCCCTGACACCAT	TGGCGATGTACTTCCCAGCA
Fdx1	ATGGCGAGACGCTAACGACC	ACATCCGCCACTGCTTCAGG
FSHR	GTGCATTCAACGGAACCCAGC	CGCCTCCAGTTTGCAAAGGC
GATA4	GAAGACACCCCAATCTCG	TTGTGATAGAGGCCACAGG
GATA6	AAACGCCGGTGCTCCACAGCTTACAGG	TGATGAAGGCACGCGCTTCTGTGGC
Grem1	AGCGAAGAACCTGAGGAC	CTCCTTGGGAACCTTTCTT
Grem2	TGTCATTCACAGAGAGGAGAG	TTCTTCCGTGTTTCAGCTAC
HSD17b1	TGTTCGCCTAGCTTCAGGATCTCC	CCACAGATTTGGAGTCTCTGACATCC
IGFBP2	TGTGAAAAGAGACGCGTGGGCA	ATGGTCCCATCCACGTGGTTCT
IGFBP4	TGCACGGAGCTGTCGGAAATC	TCCCCACGATCTTCATCTTGCTC
IGFBP5	TCAACGAAAAGAGCTACGGCGA	GGAAATGCGAGTGTGCTTGGG
Inhα	CCCACCCTTATTACTCAACACTGTGC	GGGTGGAGCAGGATATGGATCC
Inhβa	AGCTTCATGTGGGTAAAGTGGGG	GACAGGTCACTGCCTTCCTTGG
Inhβb	GTGAACCAGTACCGCATGCG	ACACTCCTCCACGATCATGTTGG
L19	CAATGAGACCAATGAAATCG	GCAGTACCCTTCCTCTTCC
LDLR	TGTTCCAAGAGGCAGGGTCC	TTGGCCACTGGATGTTTTCGG
Lhcgr	TGTAACACAGGCATCCGGACC	ACTCCAGCGAGATTAGCGTCG
Map3k5	GAAAGGCCGCCGACATTTGG	TATGAACGCCTTGGCCTCCG
OVGP1	AAGGGGAAGGAGTGGCTTGG	GTGGCAAGGGGGTTGAATTGG
Papp-a	TGTAGGTCACAGGACATAGG	TGGTCTCCAGTGGTATCC
Pax2	TCTTTGAGCGTCCTTCCTATCCC	CTCTGTGTGCCTGACACATTGC
Pla2g4a	AGACCTACGGTTCAGCATGGC	AGAGAATCCCACCATGGCCC
PlxnC1	ACTTCCTCTTTGTAGGTTGG	GGTAAACATCCTGAAGAACC
Prkar2b	GGACTAACCACTACTACGG	GTCACTAACAAACCAAGAGC
Prlr	GAGAAAAACACCTATGAATGTC	GTAAGTCACATCCACATAAAGT
Sema5a	AAGTGGTGCAGCCAGTGACC	CTCTTCCAGCAAACAGCTGCC
Sema7a	TCAGAGGGTGGAACCTATGGGG	ACCACCTTGTGAATGGTGCCC
Vcan	AGTGATGCAGGCGTCTACCG	TCTGGGCTTGCTATGACCGC

TABLE II. REAL-TIME PCR PRIMERS

Primers are listed 5’ to 3’.

per nanogram of total RNA. The results are expressed as the ratio between the copies per nanograms of total RNA of the gene of interest and ribosomal L19 protein.

F. Microarray Analysis

Total RNA was labeled and hybridized to the Affymetrix Mouse Gene 1.0 ST Array according to the protocol recommended by Affymetrix. Scanned images of each chip were analyzed for the following quality metrics: total background, raw noise, average signal present, signal intensity of housekeeping genes, 3'/5' signal ratio of housekeeping genes, relative signal intensities of labeling controls, and absolute signal intensities of hybridization controls. All hybridizations passed according to indicated quality criteria. Microarray data analyses were performed using the software package BRB Array Tools. Filter threshold values were set to a minimum value of 25. Array normalization was performed by using the median of a set of housekeeping genes as reference. ANOVA tests were used to calculate significance of the differential expression. For all genes, the fold change was calculated by dividing the mutant value by the wildtype value. If this number was less than one, the reciprocal is listed. The reported fold changes are the average of three animals for each genotype. A change was deemed significant only if the *P* value was less than 0.01 and the fold change was more than 2. Differentially regulated genes were then analyzed in Database for Annotation, Visualization and Integrated Discovery (DAVID), Gene Set Enrichment Analysis (GSEA) and Significance Analysis of Microarrays (SAM) for functional pathway analysis.

G. Western Blot Analysis

Primary mouse granulosa cells were homogenized in an ice cold radioimmune precipitation assay lysis buffer (50 mM Tris- HCl, pH 7.4; 150 mM NaCl; 1% Nonidet P-40; 0.25% sodium deoxycholate; 1 mM phenylmethylsulfonyl fluoride; 150 mM EDTA; 1X protease inhibitor cocktail (Sigma); 1 mM NaF; and 1 mM Na3VO4). Protein concentration was determined using BSA as a

standard. The samples were denatured by adding sample buffer (0.555 M bis-Tris, 4.44% sodium dodecyl sulfate, 0.333 M HCl 30% glycerol, 2.22 mM EDTA, 10% β-mercaptoethanol, 0.04% bromophenol blue), followed by boiling at 90°C for 10 min. Protein (12 μg) was separated on 12% bis-Tris- PAGE gels in 250 mM 3[*N*-morpholino]propanesulfonic acid, 250 mM Tris, 5 mM EDTA, 1 M sodium bisulfite, and 0.5% sodium dodecyl sulfate buffer. Samples were transferred to nitrocellulose membranes followed by incubation in 5% nonfat dry milk for 2 hs at room temperature to block unspecific binding.

After several washes in Tris-buffered saline-Tween 20, the membranes were incubated overnight at 4°C with anti-GATA4 (1: 500), anti-GATA6 (1:100), anti-β-actin (1:500), anti-Lamin B1 (1:500), or anti-FSHR (1:1000) antibody. Membranes were washed and incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000) for 2 hs at room temperature. Protein-antibody complexes were visualized using Immoblon western chemiluminescent horseradish peroxidase substrate (Millipore Corp., Billerica, MA) or Supersignal Westfemto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL) depending on the abundance of the protein and sensitivity of the antibody.

H. Immunohistochemistry

For chapters III and IV, ovaries were fixed in Bouin's Solution before paraffin embedding. For chapter V, ovaries, oviducts and uteri were fixed in formalin. Sections (4-5 μm) were dewaxed and rehydrated. This was followed by antigen retrieval using citrate buffer solution (10mM citric acid and sodium citrate, pH 6) and microwaved on high for 30 sec until boiling and then at low for 8 more minutes. After cooling, slides were placed in 1%H2O2. Sections were then blocked using the Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA) followed by 30 min of blocking in superblock blocking buffer (Pierce Chemical Co., Rockford, IL) before the addition of the primary antibody diluted in PBS (Chapter III: GATA4 (1:500), GATA6 (1:200), and cleaved caspase 3 (1:200); Chapter IV: cleaved caspase 3 (1:200), PCNA (1:300), and Vcan (1:300); Chapter V: GATA4 (1:25),

GATA6 (1:25), PAX2 (1:50), cleaved caspase 3 (1:200), PCNA (1:300), and VEGF (1:50)) After washes with Tris-PBS, slides were incubated in secondary antibody for 30 min at room temperature followed by washing. Tissues were stained through use of the Vectastain elite ABC kit and 3, 3´-diaminobenzidine (Vector laboratories) following manufacture's recommendations. Slides were counterstained with Gill's hematoxylin before mounting.

I. Hematoxylin and Eosin Staining (H&E)

Dewaxed and rehydrated tissue sections were dipped in Harris Hematoxylin (Fisher Scientific, Rockford, IL) followed by rinsing in running water. Slides were next dipped in Define Reagent (Fisher Scientific) followed by rinsing in running water. Next, Bluing Reagent (Fisher Scientific) was used to sharpen the staining of the nucleus followed by rinsing in running water. Slides were then placed in 95% ethanol followed by staining in Eosin-Y and Phloxine (Thermo Scientific) to stain the plasma.

J. Plasmid and Reporter Constructs and Cell Transfection

The region between -311 to -1 of the *FSHR* gene, where -1 is the transcription initiation site gene, was cloned from rat genomic DNA using the following primers: forward, GCG GTA CCA AAT ATG CAC CAA GTT TCT CTT TTC TG; reverse, GCC TCG AGC CTT ATT TAT CCA TTC ACC GAC TTT C. PCR products were cloned into the pGL4 Basic luciferase report vector (Promega Corp., Madison WI) and confirmed by sequencing. This reporter construct was named rFSHRp-Luc. The GATA-binding site found at position -82 of the FSHR promoter was mutated using QuikChange IIXLSite-Directed Mutagenesis (Stratagene, La Jolla, CA). Both wildtype and mutant rFSHRp-Luc were transfected into primary granulosa cells along with a mouse GATA4 expression plasmid or an empty plasmid (pcDNA) as previously described (9). Luciferase activity was assessed 48 h after transfection using the Dual Luciferase assay kit (Promega).

K. Gel Shift Analysis

Nuclear protein extracts were isolated from primary granulosa cells (71). Radioactive labeled double-stranded oligonucleotide probes containing the GATA-binding sites were used as probes. The shift band obtained with this probe was competed against 50x excess of unlabeled wild-type oligonucleotides or mutant oligonucleotides containing a TATC to T*gg*C mutation on the GATA-binding site. Supershift experiments were carried out by adding 2 µl of the GATA4 antibody (sc9053/H-112X), 2 µl of GATA6 antibody (sc-7245/N-18 X), or 2 µl of normal serum to the binding reaction 30 min before the addition of the labeled probe. Both GATA4 and GATA6 antibodies have been shown to be effective in supershift reactions (69). Reactions were performed as previously described (71).

L. Statistics

Data are expressed as the mean ± SEM. Multiple group statistical analyses were performed by one-way ANOVA followed by the Tukey test for multiple comparisons. Two-group comparisons were performed using a *t* test for independent samples. Statistics were calculated with GraphPad Prism 5 (GraphPad, La Jolla, CA).

III. LOSS OF GATA4 AND GATA6 IN GRANULOSA CELLS BLOCKS FOLLICULOGENESIS, OVULATION, AND FOLLICLE STIMULATING HORMONE RECEPTOR EXPRESSION LEADING TO INFERTILITY (83)

A. Introduction

Follicle selection and atresia are highly regulated pathways controlled by genes involved in differentiation, cell survival, steroidogenesis and apoptosis. Several genes included in these diverse processes contain within their regulatory regions the motif WGATAR, which is recognized by the family of transcription factors known as GATA (55-57). Of the six members of this family only GATA4 and GATA6 are highly expressed in ovarian granulosa cells (56,61,62). Both GATA4 and GATA6 have been shown to regulate the expression of genes involved in follicle growth and steroid synthesis (55). GATA4, in particular, mediates the stimulatory effects of FSH on several ovarian genes (56). For instance, silencing of GATA4 in primary rat granulosa cells blunts the stimulation of aromatase by FSH (71). Likewise, mutation of a GATA-binding site on the steroidogenic acute regulatory protein (StAR) promoter prevents both basal and FSH-driven activity (84), whereas forced expression of GATA4 in porcine granulosa cells increases StAR promoter activity (56,69). GATA4 and GATA6 can also activate the type II 3β-hydroxysteroid dehydrogenase promoter (79). Overexpression studies have demonstrated that GATA4 activates the promoter of ovarian hormones including inhibin-α (70) and anti-Müllerian hormone (85), both of which are produced by the granulosa cells (86,87). This evidence suggests that GATA4 and GATA6 may have a crucial role in the regulation of granulosa cell function. In fact, a recent report demonstrated that conditional deletion of exons 3, 4, and 5 of the *GATA4* gene in reproductive tissues results in impaired fertility (88). In addition, transgenic mice expressing a small interfering RNA against GATA4 develop ovarian tumors, suggesting that this factor is also involved in the function and integrity of the ovary (89). The role of GATA6 in the regulation of ovarian function *in vivo*, however, has yet to be examined.

GATA4 and GATA6 are coexpressed in granulosa cells, recognize identical binding motifs, and regulate the expression of similar target genes (67,90-92). Therefore, we hypothesize that these factors

have redundant functions in the ovary and that loss of both GATA4 and GATA6 results in a significant decrease in ovarian function. In this chapter, we test this hypothesis and investigate the roles of GATA factors in folliculogenesis by silencing the expression of GATA4, GATA6, or GATA4 and GATA6 in granulosa cells using a Cre recombinase (Cre)-Lox system.

B. Results

1. Granulosa Cell-specific Disruption of the *GATA4* and *GATA6* Genes

Cre-Lox technology was used to knock down GATA4 and GATA6 expression in granulosa cells. Exon II of the *GATA4* and *GATA6* genes was targeted for deletion because it codes for the majority of both GATA proteins (93,94). To maximize the recombination of the floxed genes, we generated animals carrying one floxed allele and one null allele (F/-) using zona pellucida glycoprotein-3 Cre (Zp3-Cre) animals (95). GATA4F/- and GATA6F/- animals are fertile and have normal ovarian structure and function. To selectively disrupt GATA expression in granulosa cells, we initially attempted to use mice expressing Cre driven by the Anti-mullerian hormone receptor (AMHR) promoter. However, to our surprise, animals carrying AMHR-Cre alone had reduced fertility compared to nontrangenic animals (Fig. 6). Therefore, we used a recently developed transgenic mouse that expressed Cre under control of the proximal promoter (PII) of the aromatase gene (*Cyp19*) (81).

Cyp19-Cre mice had fertility comparable to controls (Fig. 6). In Cyp19-Cre animals, Cre is expressed in granulosa cells of antral follicles and has not been detected in the theca, epithelial cells, or the oocyte throughout postnatal development (81). In this study, we used GATA4F/-; Cyp19-Cre ($G4^{gcko}$), GATA6F/-; CYP19-Cre ($G6^{gcko}$), or GATA4F/-GATA6F/F; CYP19-Cre ($G4/6^{gcko}$) animals in all experiments unless a specific genotype is indicated. Wildtype and GATAF/+; Cyp19-Cre animals were used as controls.

Immunostaining for GATA4 and GATA6 in $G4^{gcko}$ and $G6^{gcko}$ ovaries confirmed GATA knockdown in granulosa cells (Fig. 7A). Strong staining for GATA4 was found in $G6^{gcko}$ ovaries (used

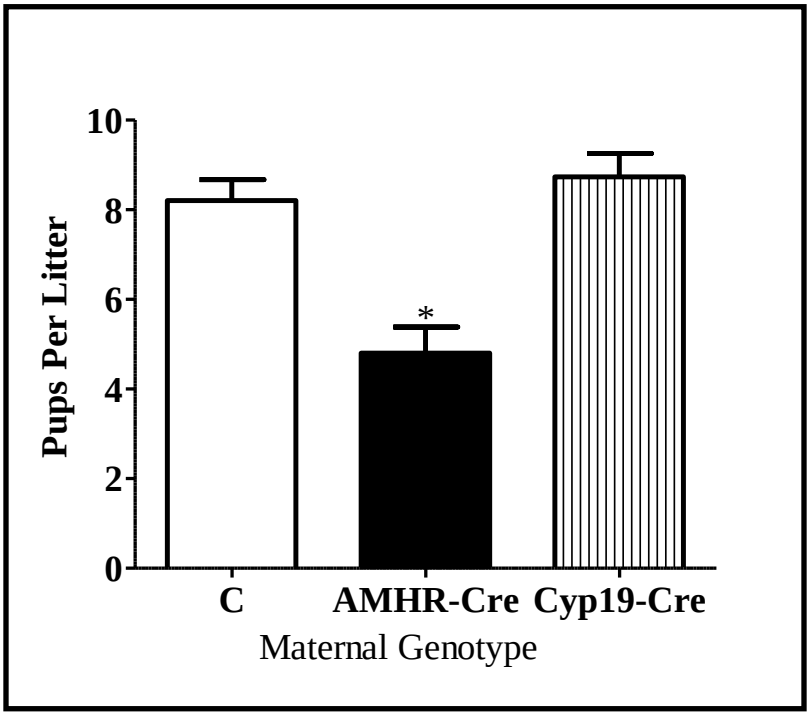


Figure 6. AMHR-Cre Females are Subfertile Compared to Cyp19-Cre and Wildtype Females.

The number of pups per litter was compared between wildtype control (C), AMHR-Cre, and Cyp19-Cre female mice. None of these females carried floxed genes. All females were paired with proven fertile males. At least two different females were used in obtaining the average number of pups per litter. *Columns* represent the mean ± SEM. *Asterisk* (*) denotes significance compared to control (*, $P<0.05$).

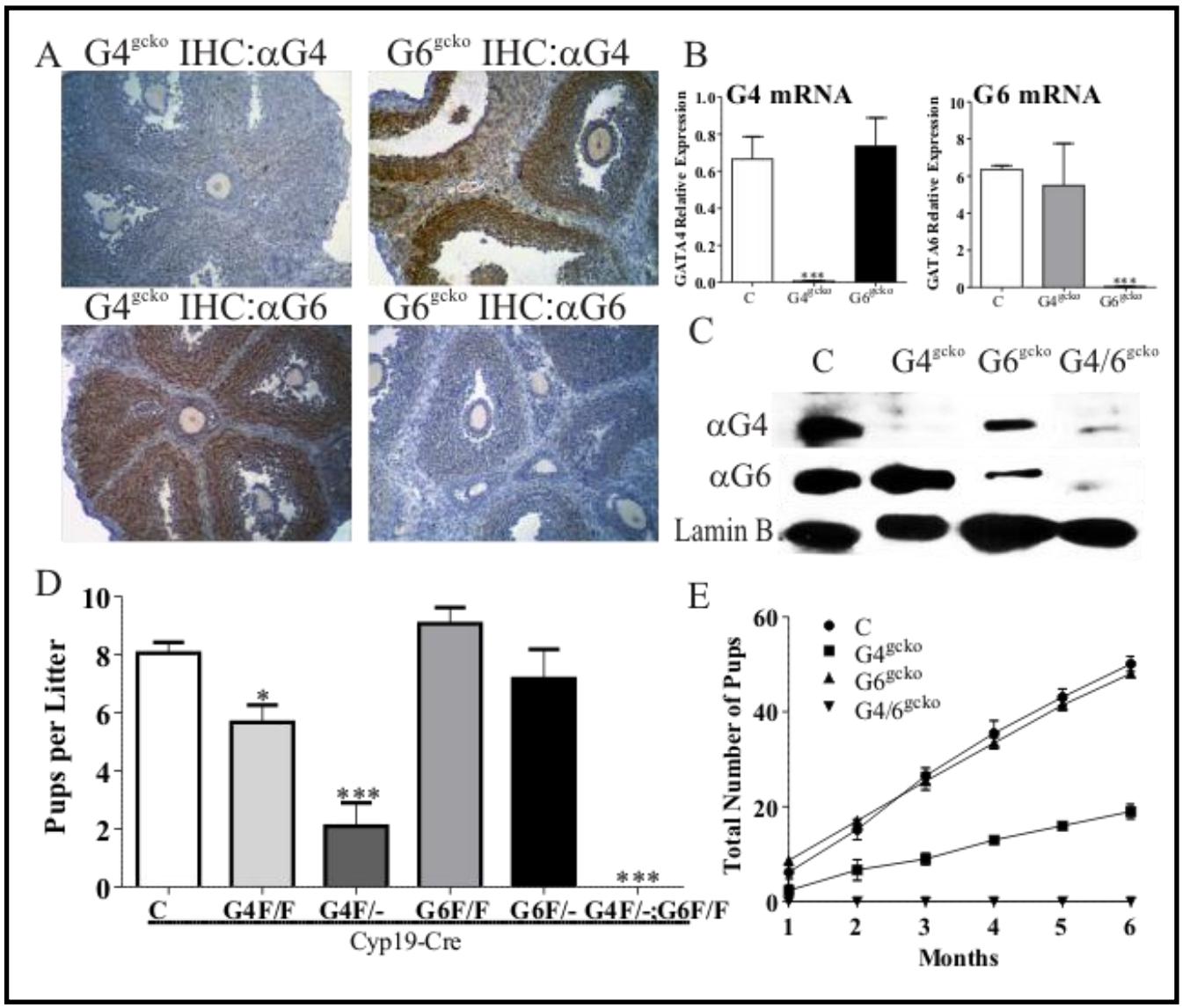


Figure 7. GATA4 and GATA6 Expression in Granulosa Cells is Necessary for Normal Fertility.

A) 10x magnification of IHC for GATA-4 protein in G4^{gcko} (*top left*) ovary compared with GATA4 protein in the G6^{gcko} (*top right*) ovary from d21–25 eCG-stimulated mice. GATA6 protein expression in G4^{gcko} (*bottom left*) ovary compared with in G6^{gcko} (*bottom right*) ovary (n = 3 for each genotype; only a representative is shown). **B**) mRNA expression of GATA4 (*left*) and GATA6 (*right*) from eCG-treated animals. Expression is expressed relative to mouse ribosomal L19 (the average of six or more samples per genotype is shown). ***, $P < 0.01$. **C**) Protein expression of GATA4 (*top*) and GATA6 (*middle*) from eCG- treated females. Lamin B1 was used as a loading control (*bottom*). A representative experiment of three or more samples for each genotype is shown. **D**) The number of pups per litter was determined in control mice (C) and experimental animals (G4F/F; G4F/-; G6F/F; G6F/- and G4F/-; G6F/F). All animals carried the Cyp19-Cre expression cassette. Four or more females were used for each genotype. *Columns* represent the average ± SEM of the number of pups per litter. *Asterisk* denotes significance compared with control (*, $P < 0.05$; ***, $P < 0.01$). **E**) Continuous breeding assessment showing the cumulative number of progeny per female. Values represent the mean ± SEM of litters derived from three or more females for each genotype. IHC, immunohistochemistry.

as control), whereas little or no staining was observed in $G4^{gcko}$. The opposite findings were observed when staining for GATA6 was performed.

The knockdown of GATA4 and GATA6 at the level of mRNA was confirmed using granulosa cells isolated from preovulatory follicles. As shown in Fig. 7B, GATA4 mRNA levels were undetectable in granulosa cells obtained from $G4^{gcko}$ animals, whereas no differences in GATA4 expression were found between control and GATA6 knockout cells. Similarly, GATA6 mRNA was undetectable in granulosa cells from $G6^{gcko}$ animals, but it remained highly expressed in control and GATA4 knockout cells. Western blot (Fig. 7C) confirmed these results and showed very low levels of GATA4 protein in GATA4 knockout cells as well as in the double GATA4 and GATA6 knockout cells. GATA6 protein levels in single and the double knockout animals were significantly lower than in control and $G4^{gcko}$ mice.

2. GATA4 and GATA6 are Essential for Female Fertility

The fertility of mice lacking GATA4 and/or GATA6 in granulosa cells was tested by mating control or experimental ($G4^{gcko}$, $G6^{gcko}$ or $G4/6^{gcko}$) females with males of proven fertility for 6 months. No differences were found in the number of pups per litter between control and $G6^{gcko}$ (Fig. 7D); however, both G4F/-; Cyp19-Cre and G4F/F; Cyp19cre animals had a significant decrease in the number of pups per litter when compared with controls. Fertility defects were more significant in the G4F/-; Cyp19-Cre females, which produced significantly less pups per litter than G4F/F. In addition, six of 11 (54%) G4F/-; Cyp19-Cre females failed to produce offspring, suggesting that the reproductive capacity of G4F/-; Cyp19-Cre females ranges from subfertility to infertility. In marked contrast, all double-knockout females were infertile. The total number of pups produced by females of each genotype was monitored over the course of 6 months. As shown in Fig. 7E, no differences in the progressive accumulation of pups were observed between control and $G6^{gcko}$ animals. In contrast, the total number of pups produced by $G4^{gcko}$ females was significantly lower whereas $G4/6^{gcko}$ females

produced no pups. These results demonstrate that the expression of both GATA4 and GATA6 in granulosa cells is essential for female fertility.

Ovarian granulosa cells play a central role in the regulation of the murine estrous cycle; therefore, we next compared the length of each phase of the estrous cycle in control, $G4^{gcko}$, $G6^{gcko}$, and $G4/6^{gcko}$ animals. The results showed that $G4^{gcko}$ and $G4/6^{gcko}$ have significantly fewer complete cycles when compared with control (C) and $G6^{gcko}$ mice (Fig. 8). Accordingly, the average number of days in proestrus was significantly decreased in $G4^{gcko}$ and $G4/6^{gcko}$ animals, suggesting a defective follicular phase. There was also a decrease in the days that GATA4/6 mutant animals spent in estrus when compared with all the other phenotypes, although this was not statistically significant. Accordingly, $G4/6^{gcko}$ animals stayed in metestrus/diestrus for an extended period. Based on these results, it is not surprising that estradiol and progesterone levels were highly variable in $G4^{gcko}$ animals. Because of this variability, no significant differences in the serum levels of these steroids were found between control, $G4^{gcko}$, and $G6^{gcko}$. However, estradiol levels were significantly lower in $G4/6^{gcko}$ mice when compared with control animals (Fig. 8). On the other hand, gonadotropin levels (FSH and LH) were significantly higher in $G4/6^{gcko}$ mice when compared with all other genotypes including control. Taken together, these results show that $G4^{gcko}$ and $G4/6^{gcko}$ animals have abnormal estrous cycles, suggesting impaired folliculogenesis and/or irregular ovulation.

To investigate whether folliculogenesis was defective, ovaries of adult 50- to 70-days-old animals at proestrus were microscopically examined (Fig. 9A). This study indicated that the ovarian morphology of $G4^{gcko}$ and $G6^{gcko}$ animals appears normal, showing the presence of follicles at all stages of development and corpora lutea (n =3 for each genotype). In marked contrast, ovaries of double $G4/6^{gcko}$ animals contained only few small antral follicles and no large/preovulatory follicles nor corpora lutea, suggesting that both GATA6 and GATA4 factors are needed for normal folliculogenesis and luteal formation. The presence of large antral follicles in control, $G6^{gcko}$, and $G4^{gcko}$ animals was confirmed at higher magnification (x20) (Fig. 9B and data not shown). At this

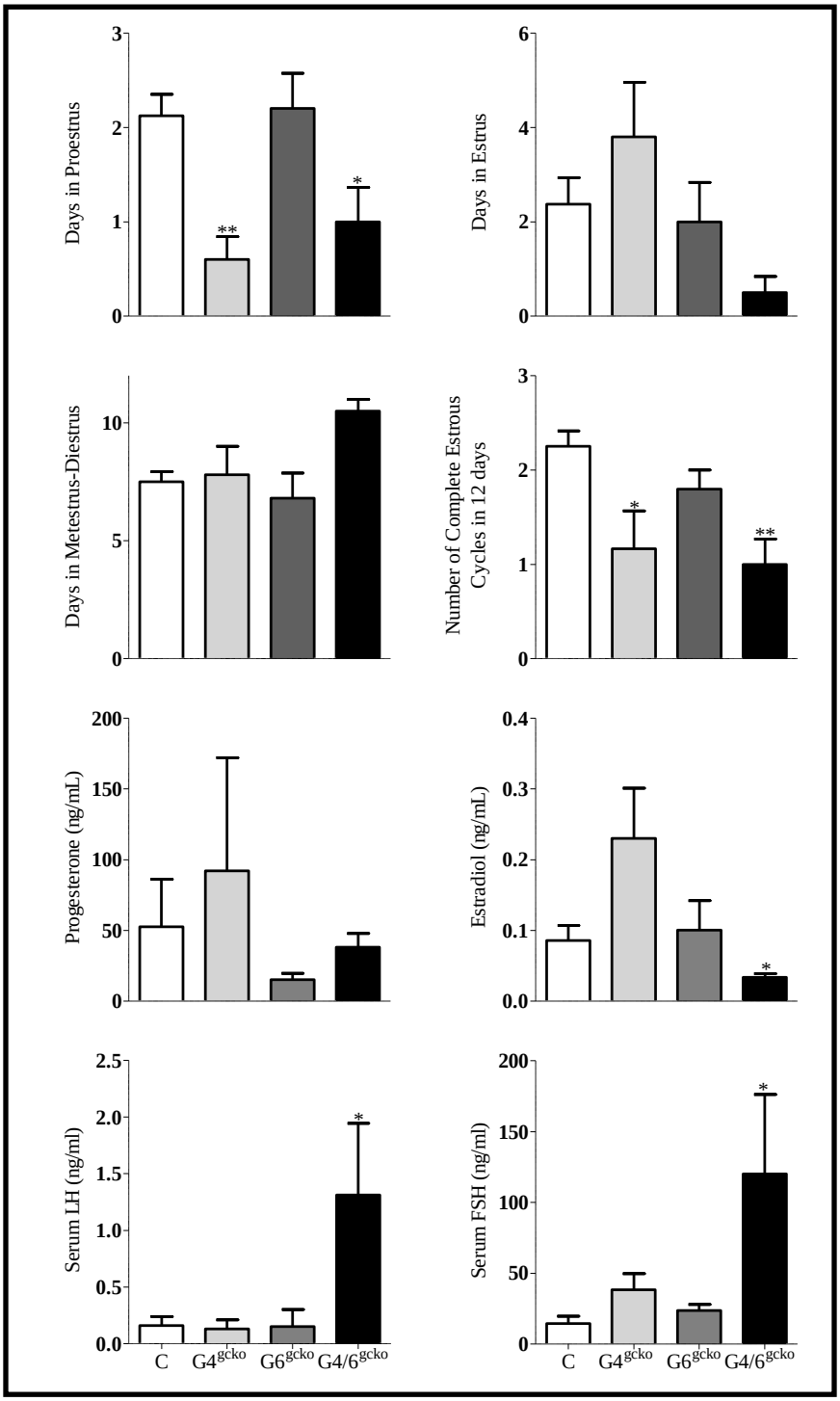


Figure 8. Abnormal Estrous Cycling in G4^{gcko} and G4/6^{gcko} Mice.

The estrous cycle was tracked over the course of 12 d in control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} adult females. Each estrous cycle was broken into three stages of proestrus (P), estrus (E), and metestrus/diestrus (M/D). The *columns* represent the average number of days spent at each stage ± SEM. Estradiol, progesterone, LH, and FSH levels were determined at the proestrus stage. *Bars* represent the mean ± SEM. N ≥ 4 animals per genotype. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

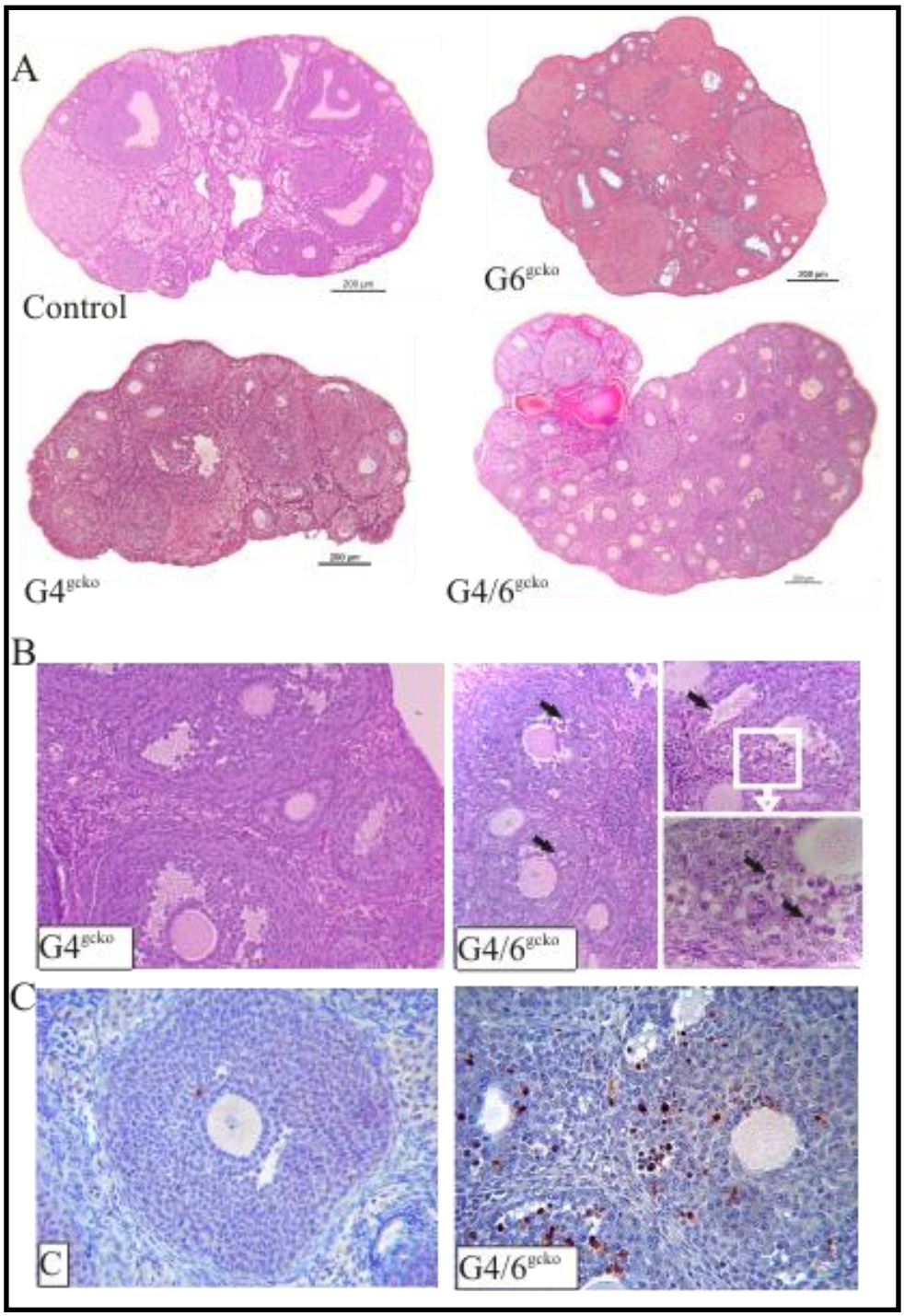


Figure 9. Abnormal Folliculogenesis in G4/6^{gcko}.

A) Representative hematoxylin and eosin staining of ovaries of control, G4^{gcko}, G6^{gcko} and G4/6^{gcko} (average age 60 d) females at proestrus. **B**) Follicles in G4/6^{gcko} animals show morphological characteristics of atresia. Hematoxylin and eosin staining of representative ovaries from G4^{gcko} and G4/6^{gcko} animals at a 20x magnification. High magnification (X40) is included for G4/6^{gcko} animals. *Arrows* point to granulosa cells with pyknotic nuclei, which were found only in G4/6^{gcko} ovaries. Control and G6^{gcko} animals showed no sign of atresia (data not shown). **C**) Cleaved caspase 3 staining in control and G4/6^{gcko} animals. N=4 but only one representative picture from each genotype is shown.

magnification, it was also evident that the granulosa layer of G4/6^{gcko} follicles shows morphological characteristics of atresia (96,97) including granulosa cells with pyknotic nuclei (Fig. 9B, *right panel*).

Furthermore, G4/6^{gcko} follicles showed increased staining for cleaved caspase 3 (Fig. 9C, *right panel*), which is known to be a key executioner of apoptosis (98). Negligible cleaved caspase 3 staining was observed in controls (Fig. 8C, *left panel*). These results confirm the abnormal follicular development suggested by the acyclicity of mice lacking GATA6 and GATA4 in granulosa cells and demonstrate an increase in follicular atresia in these animals. Based on these results, we examined whether follicle growth in G4/6^{gcko} animals could be rescued by treatment with exogenous gonadotropin. Immature (d 21–d 25) mice received a single dose of eCG and killed 48 hs later to collect ovaries for histological analysis. Control and single knockout mice responded to eCG stimulation by producing a large number of antral follicles (Fig. 10A). Hemorrhagic follicles were frequently found in G4^{gcko} but rarely in G6^{gcko} ovaries. No hemorrhagic follicles were found in control animals. Strikingly, the ovaries of G4/6^{gcko} animals did not contain antral or preovulatory follicles after eCG treatment and weighed less than control, G4^{gcko}, and G6^{gcko} ovaries (Fig. 10B). These results demonstrate that follicular development cannot be rescued by exogenous gonadotropins in conditional GATA-knockout animals.

After eCG treatment, progesterone levels were not significantly different between knockouts and control animals (one-way ANOVA, Fig. 10C); although there was a trend suggesting lower progesterone levels in double-knockout animals. In fact, when progesterone levels in control and double-knockout animals were analyzed separately using a *t* test they were found to be significantly different ($P < 0.05$). On the other hand, both G4^{gcko} and G4/6^{gcko} females had a significant reduction in serum estradiol levels when compared with control and G6^{gcko} (Fig. 10D), suggesting that estrogen production in the ovary is mainly regulated by GATA4.

Because follicle growth is defective in G4/6^{gcko} mice, the response of control and mutant animals to a superovulation protocol was examined. Adult (average age 90d) control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko}

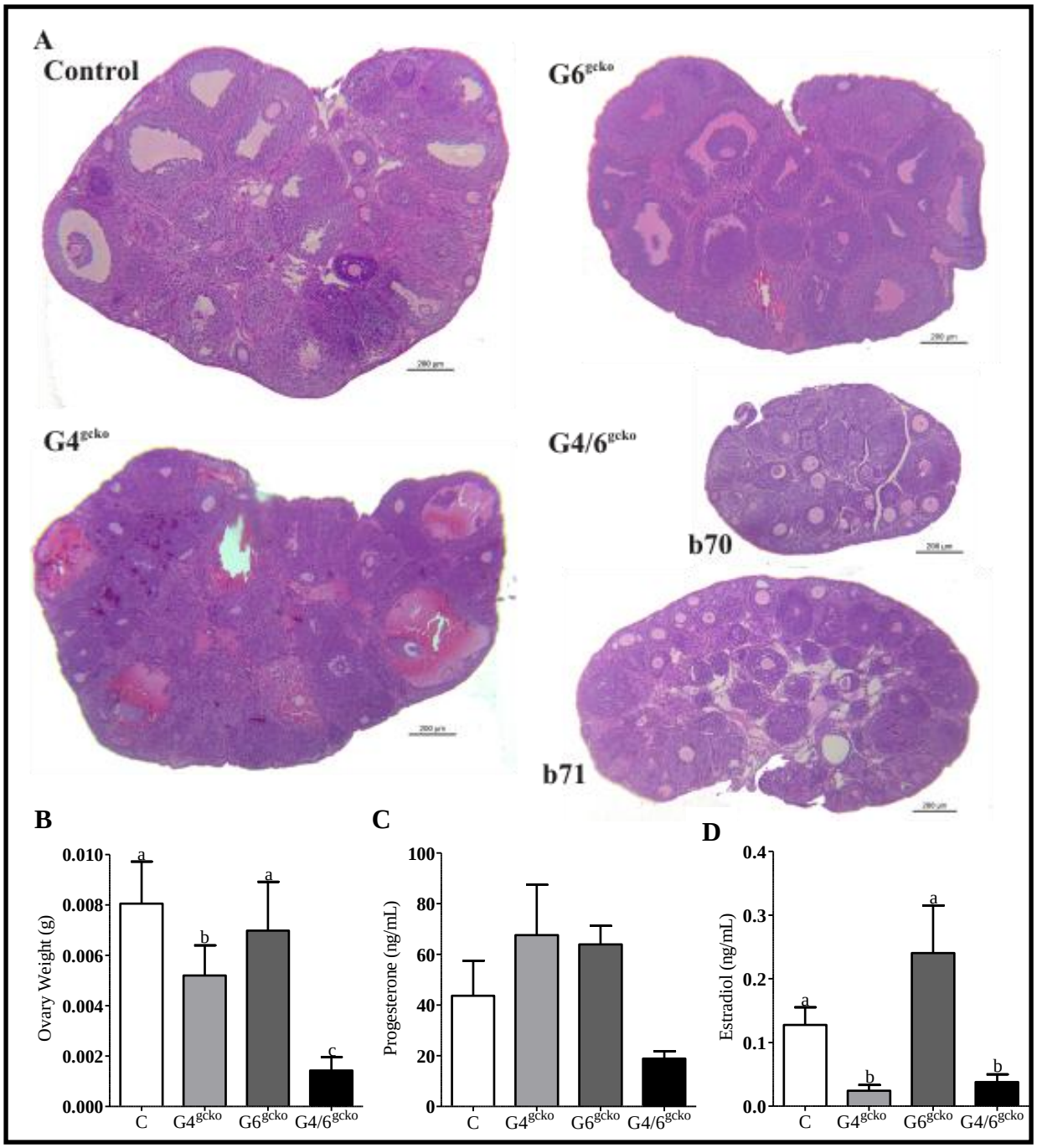


Figure 10. Effect of eCG Treatment on Follicle Development.

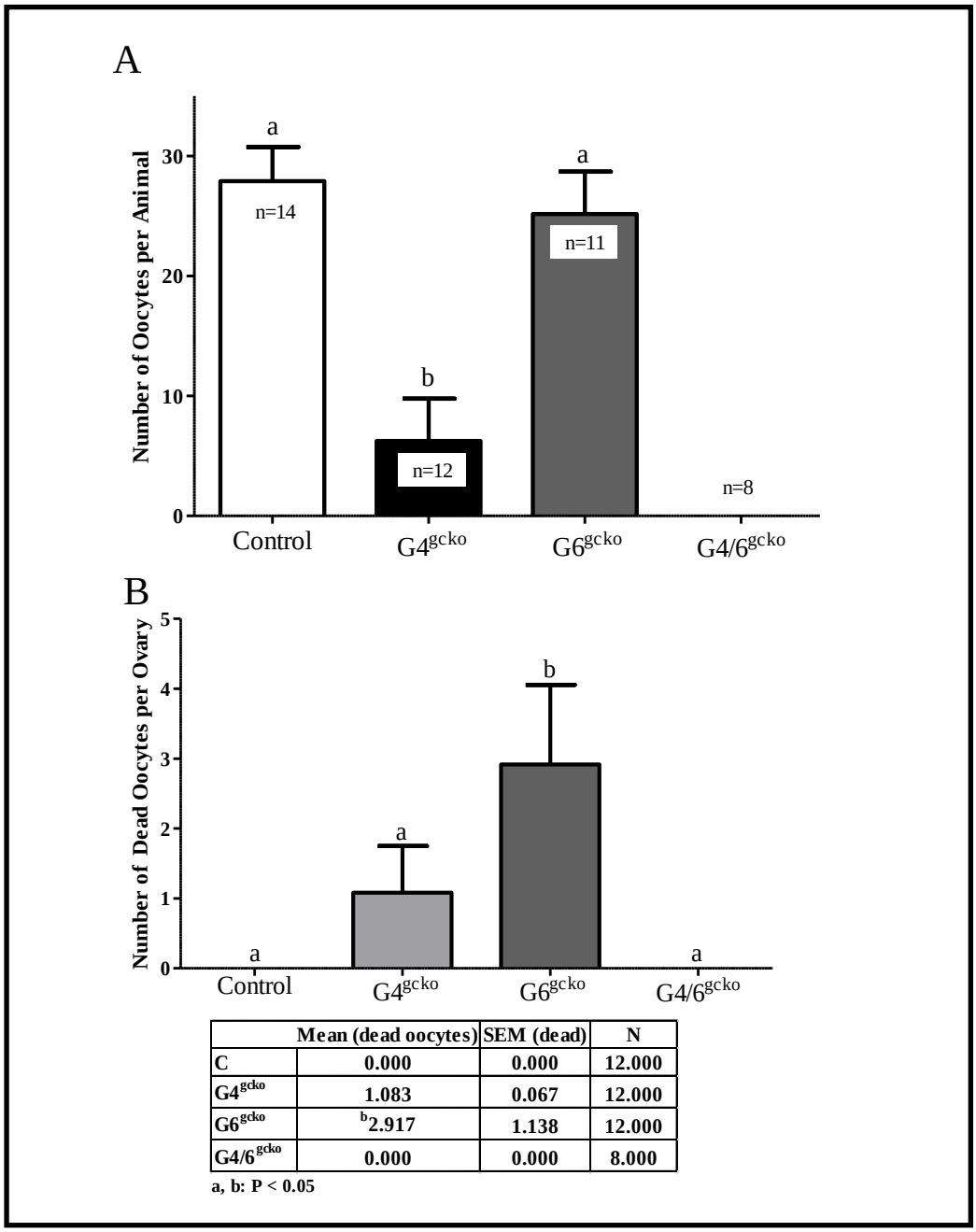
A) Representative hematoxylin and eosin staining of ovaries from animals treated with eCG for 48 hs. Four or more animals were used per group; a representative ovary is shown for control, $G4^{gcko}$ and $G6^{gcko}$ animals. The variation of two different $G4/6^{gcko}$ animals (b70 and b71) is shown. **B**) Ovarian weight; **C**) progesterone, and **D**) estradiol levels, all after 48 hs treatment with eCG. Seven or more animals were included in each group. *Bars* represent mean ± SEM, and *different letters* denote differences between groups (a and b and b and c, $P < 0.05$; a– c, $P < 0.01$).

females were stimulated with eCG for 48 hs followed by hCG administration. The presence of oocytes in the oviducts was determined 17 hs after hCG treatment. Control and G6^{gcko} females released a similar amount of oocytes: 27.9 ±2.8 and 25.2 ± 3.5, respectively (Fig. 11A). Noteworthy, approximately 12% of the oocytes recovered from the G6^{gcko} animals were dead; in contrast, no dead oocytes were retrieved from the control animals. Despite this finding, the fertility of G6^{gcko} animals was not affected (Fig. 11B). The average number of oocytes produced by G4^{gcko} females was significantly lower when compared with control or G6^{gcko} animals. Remarkably, in six of nine (66%) G4^{gcko} females, ovulation did not occur. In marked contrast, all double-knockout G4/6^{gcko} females (n =8) failed to ovulate. Thus, the reduced fertility of G4^{gcko} and the infertility of G4/6^{gcko} animals correlates with impaired or lack of ovulation, respectively and does not appear to be a result of oocyte viability.

Although G4/6^{gcko} animals were unable to ovulate, we were interested to know if their oocytes were viable. We stimulated control, G4^{gcko}, G6^{gcko}, and G4/6^{gcko} with 7.5IU eCG and then punctured large follicles. Upon oocyte isolation, 70% of the oocytes were denuded in the G4/6^{gcko} in contrast to 70% of oocytes surrounded by cumulus cells in the other animals (Fig. 12A). We then went on to culture the oocytes obtained from these animals overnight to assess if maturation was affected. All knockout animals had a reduced number of oocytes at the MII stage compared to control. The number of GV, MII or oocytes in between GV/MII stages was equivalent amongst all the animals (Fig. 12B). These results suggest that GATA factors are needed for proper oocyte maturation; however further studies need to be conducted.

3. Lack of GATA4 is Associated with a Decrease in FSHR Expression in Granulosa Cells

In view of the elevated levels of gonadotropins found in the G4/6^{gcko} and the impaired response of both G4^{gcko} and G4/6^{gcko} to eCG/hCG, we examined the responsiveness of granulosa cells to gonadotropins by determining the expression of the receptors for FSH and LH in granulosa cells. The expression of aromatase, StAR, and Cyp11a1, which are known to increase during the differentiation of



	Mean (dead oocytes)	SEM (dead)	N
C	0.000	0.000	12.000
G4^{gcko}	1.083	0.067	12.000
G6^{gcko}	^{b}2.917	1.138	12.000
G4/6^{gcko}	0.000	0.000	8.000

a, b: P < 0.05

Figure 11. G4^{gcko} and G4/6^{gcko} Superovulated Females have Impaired Ovulation.

A) Defective or lack of ovulation in G4^{gcko} and G4/6^{gcko} mice, respectively. Ovulation was induced in immature (d 22– d 23) animals by a sc injection of 7.5 IU of eCG, followed 48 hs later by administration of 7.5 IU of hCG. Oviducts and ovaries were harvested 17 hs after hCG; oocytes found in the oviducts at this time were counted (n = number of animals included in each group). *Bars* represent mean ± SEM, and *different letters* denote differences between genotypes (a and b, $P < 0.05$). No oocytes were found in the oviducts of G4/6^{gcko} animals. **B**) Mean number of dead oocytes per ovary ±SEM was calculated. Oocytes were labeled as dead if they had an irregular shape or darkened nuclei. N denotes the number of ovaries used. a, b denote statistical significance in relation to control animals ($P< 0.05$)

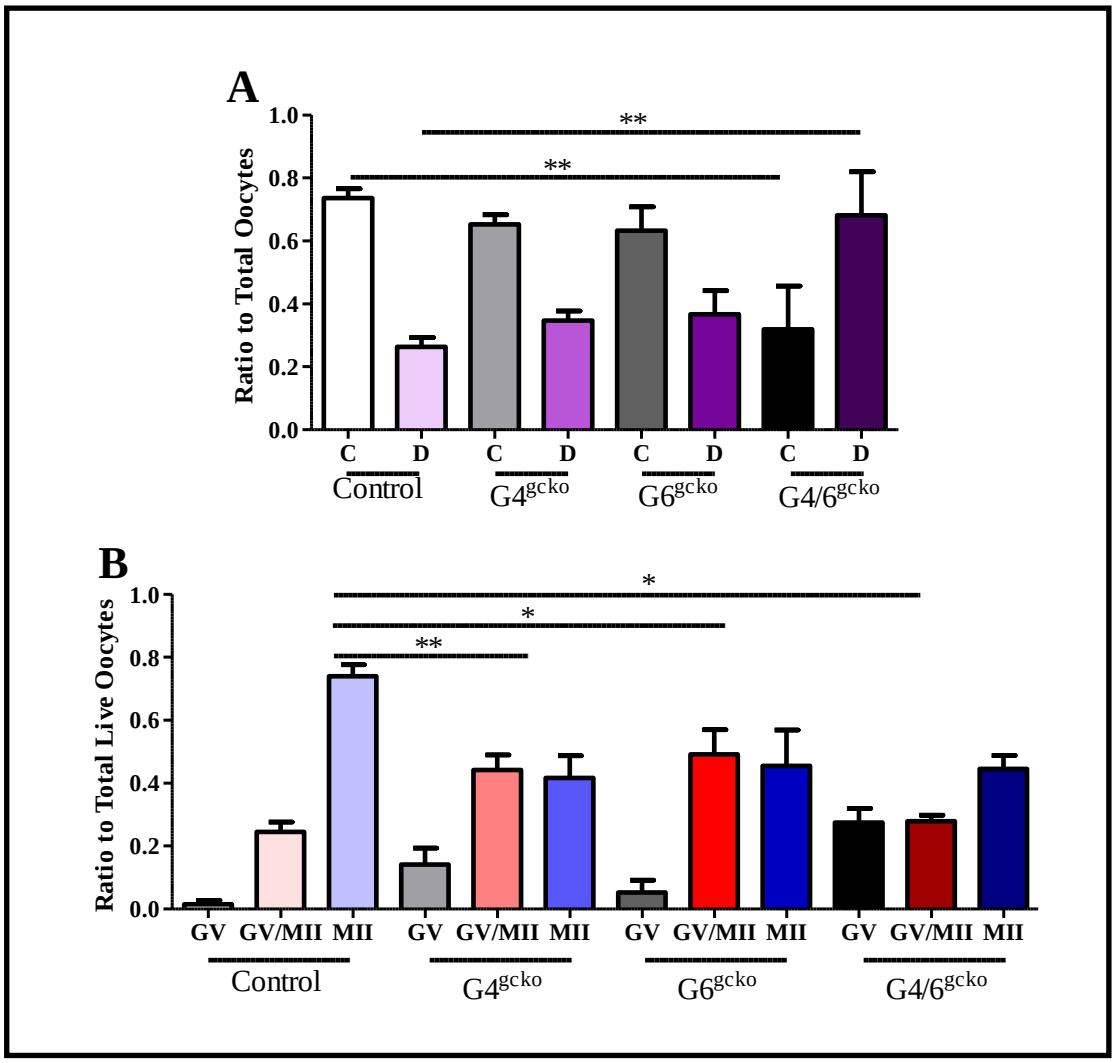


Figure 12. GATA Knockdown Impairs Oocyte Maturation

A) Oocytes were collected from control, G4^{gcko}, G6^{gcko} and G4/6^{gcko} animals by puncturing large follicles from eCG stimulated immature (~d23) animals. A) *Columns* denote the mean ratio of oocytes ± SEM that were surrounded by cumulus cells (C) or denuded (D) from the total number of oocytes collected. (**, $P < 0.01$). **B**) Maturation after overnight culture was determined in oocytes collected. *Columns* denote mean ratio ± SEM of oocytes at germinal vesicle (GV) stage, GV/ meiosis II (GV/MII) stage or meiosis II (MII) stage. N≥ 3 animals for each genotype for both experiments. (*, $P<0.05$, **, $P<0.01$)

granulosa cells, was also examined. Granulosa cells were isolated from large antral follicles of immature (d 21–d25) single-knockout and control female mice treated with eCG (7.5 IU) for 48 hs. Double-knockout animals do not produce large antral follicles in response to eCG; however, primary granulosa cells were obtained from large secondary follicles for comparison with control or single mutant animals.

As shown in Fig. 13, treatment with eCG increased FSH receptor (FSHR) mRNA expression levels in control and $G6^{gcko}$ animals. *Fshr* stimulation was prevented by the lack of GATA4 expression and by the lack of both GATA4 and GATA6. As expected, a strong induction of the LH receptor (Lhcgr), a key target of FSH in the granulosa cells, was observed in control and $G6^{gcko}$ granulosa cells but not in cells lacking GATA4 or both GATA4 and GATA6. Moreover, the expression of the classical marker of granulosa cell differentiation, aromatase (Cyp19a1) was down-regulated in $G4^{gcko}$ and $G4/6^{gcko}$ mice, suggesting that the differentiation of granulosa cells to the preovulatory stage is impaired. The stimulation of Cyp11a1 by eCG observed in control, $G4^{gcko}$, and $G6^{gcko}$ animals did not occur in the double knockout $G4/6^{gcko}$ mice. These changes in aromatase and Cyp11a1 expression are in good agreement with the serum level of estradiol and progesterone described in Fig. 10, B and C. The stimulation of StAR by eCG was not affected by GATA4 or GATA6 knockdown (data not shown).

Confirming qPCR results, Western blot analyses showed that the FSHR protein was undetectable in GATA4-knockout granulosa cells but remained highly expressed in granulosa cells of control and $G6^{gcko}$ animals (Fig. 14A). As expected, FSHR protein was nondetectable in granulosa cells of $G4/6^{gcko}$ mice (data not shown). These *in vivo* results demonstrate that GATA4 is necessary to maintain FSHR expression in the ovary. This conclusion is supported by the presence of an inverted GATA response element (GATARE) in the *Fshr* promoter (Fig. 14B).

Next, we examined whether either GATA4 or GATA6 interact with this GATA-RE using gel shift assays. A prominent shift band was observed when labeled oligonucleotides containing the GATA-RE found in the *Fshr* promoter and nuclear extracts of primary granulosa cells were used (Fig. 14C). The addition of an antibody against GATA4 to the binding reaction caused the formation of a supershift band

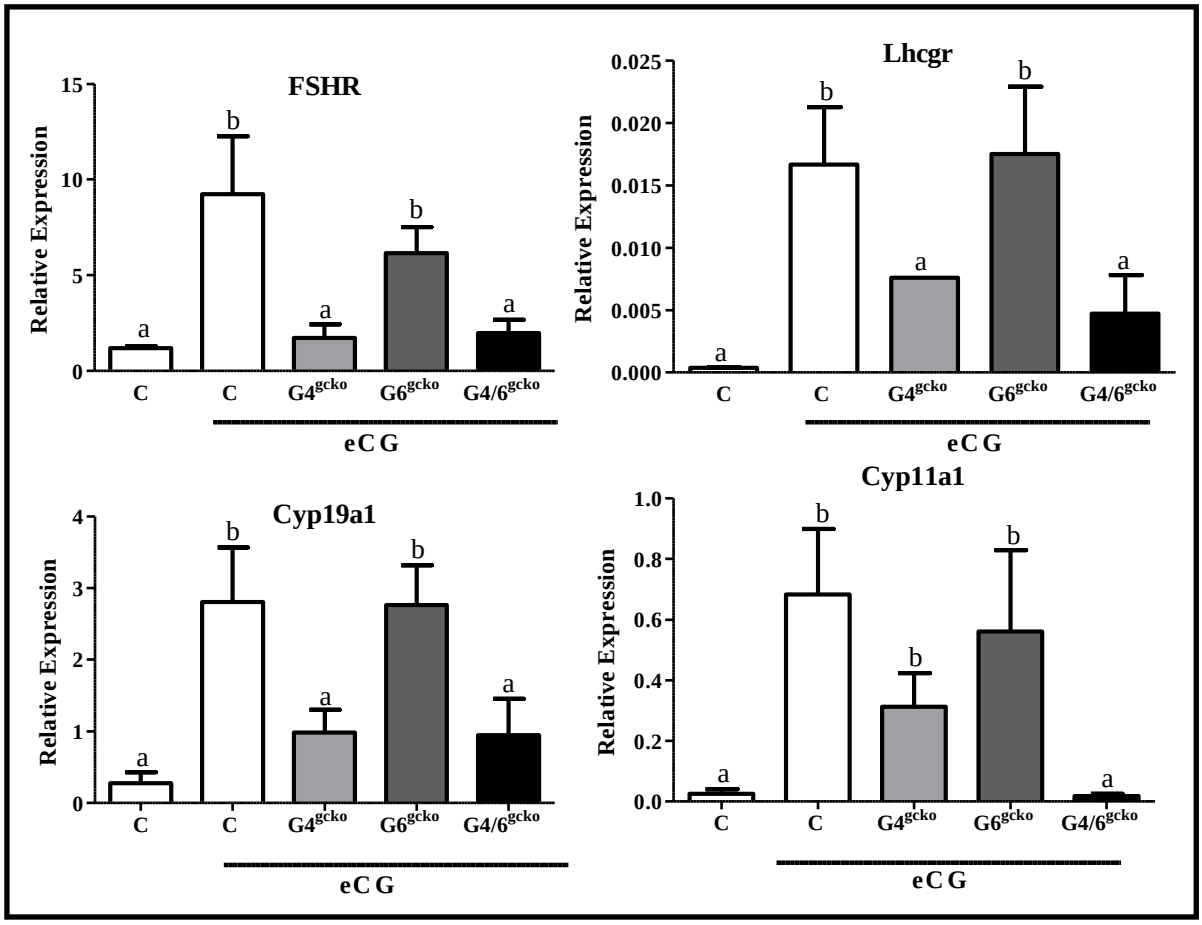


Figure 13. Relative Expression of Key Ovarian Genes in the Different Genetic Backgrounds

Total RNA was extracted from granulosa cells isolated after eCG treatment by puncturing only large antral follicles in control, $G4^{gcko}$, and $G6^{gcko}$ females or only large secondary follicles in $G4/6^{gcko}$ mice. N≥3 animals for each genotype. *Columns* represent the mean ± SEM. *Different letters* denote significance (a and b, $P < 0.05$; a–c, $P < 0.01$).

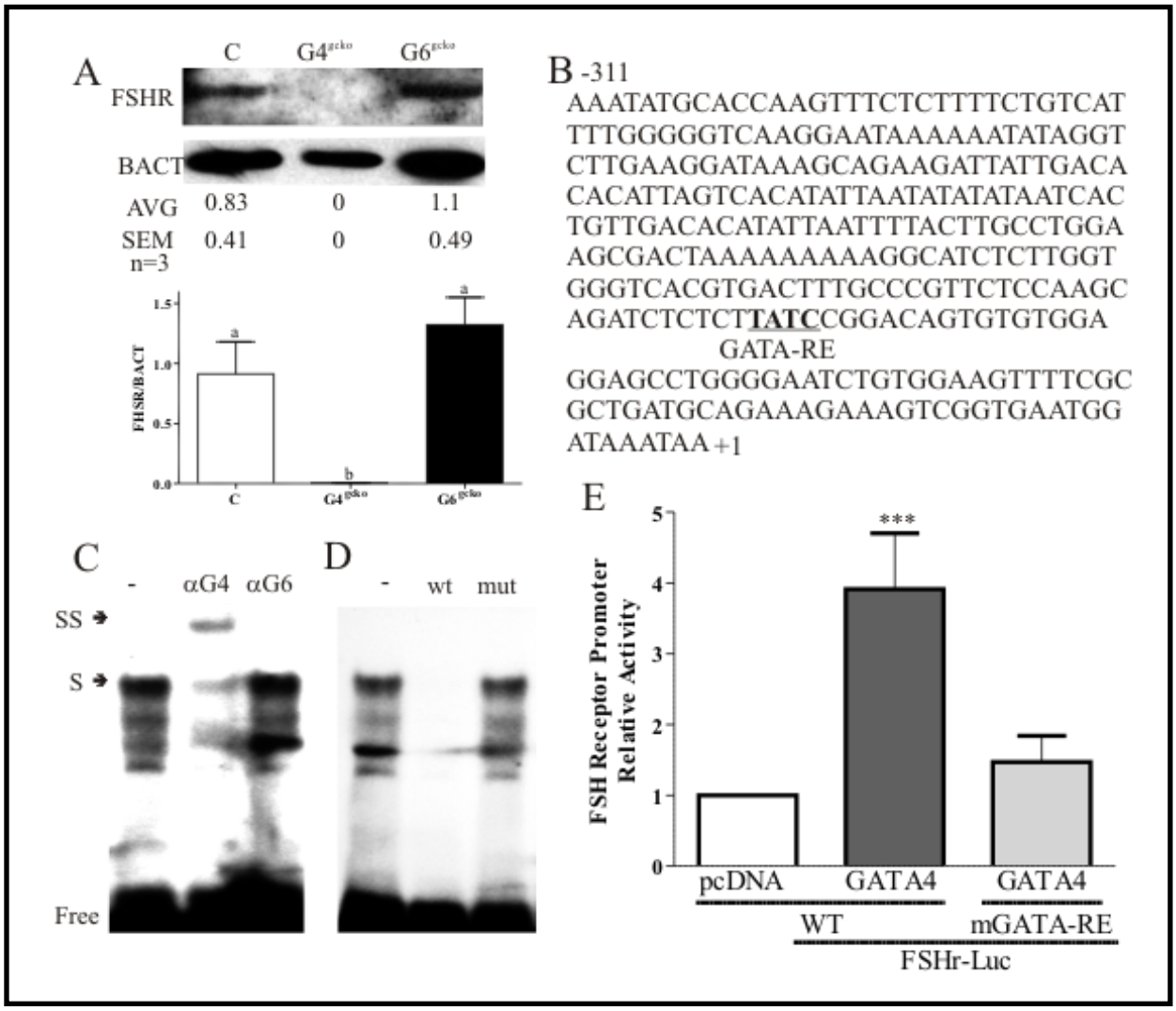


Figure 14. GATA4 regulates FSHR expression

A) FSHR protein (FSHR) levels in granulosa cells of control, $G6^{gcko}$, and $G4^{gcko}$ animals. BACT, β-actin protein. FSHR/BACT intensity ratio, SEM: SE, n = 3, *Columns* with *different letters* differ significantly (a and b, $P < 0.001$). **B**) Sequence of the proximal promoter of the *Fshr* gene indicating the location of a putative inverted GATA binding motif (GATA-RE). **C**) Gel shift assay using labeled oligonucleotides containing the GATA-RE and nuclear extracts of primary granulosa cells in the presence of normal serum (NS), an anti-GATA4 antibody (αG4), or an anti-GATA6 antibody (α-G6). **D**) Gel shift assay using the nuclear extracts from primary granulosa cells in the presence of a 50-fold excess of unlabeled wildtype (wt) probe or 50-fold excess of unlabeled mutant (mut) probe. **E**) Primary granulosa cells were transfected with a wildtype (WT) *Fshr* promoter reporter construct (FSHr-Luc) or an *Fshr* promoter construct with a mutated GATA-RE (mGATA-RE) in addition to a mouse GATA4 expression vector (GATA4) or an empty vector (pcDNA). Luciferase activity was determined 48 hs after transfection. *Bars* represent the mean ± SEM (n = 4). ***, P <0.01 vs. GATA4 or pcDNA. AVG, Average.

demonstrating that GATA4 binds to the GATA-RE oligonucleotide. The presence of an anti-GATA6 antibody in the gel shift reaction, however, did not affect the mobility of the shift band, suggesting that GATA6 is not part of the complex formed between granulosa cell nuclear proteins and the GATA-RE oligonucleotide. Moreover, the addition of a 50-fold excess of unlabeled wildtype oligonucleotide prevented the formation of the GATA4/DNA complex whereas the addition of a 50-fold excess of oligonucleotides carrying a mutation on the GATA RE (TATC to T*gg*C) had no effect (Fig. 14D). These results demonstrate that GATA4 recognizes the GATA-RE found in the *Fshr* promoter, further suggesting a role of this transcription factor in the regulation of *Fshr* expression in granulosa cells.

To investigate whether GATA4 regulates the activity of the *Fshr* promoter, this regulatory region was cloned into a luciferase reporter vector (pGL4). The *Fshr* promoter reporter (FSHr-Luc) was transfected into primary granulosa cells simultaneously with either an empty vector (pcDNA) or a GATA4 expression vector. GATA4 overexpression stimulated the activity of the FSHr-Luc reporter vector in a dose-dependent manner (data not shown). The stimulation of the *Fshr* promoter activity by GATA4 was prevented by the mutation of the GATA-RE (Fig. 14E). These results demonstrate that the expression of GATA4 alone is enough to stimulate the transcriptional activity of the *Fshr* promoter and that this stimulatory effect is mediated by the GATA-RE found in this promoter.

C. Discussion

The transcription factors GATA4 and GATA6 are expressed in the granulosa cells of growing follicles (56,61,62), suggesting that they could play a crucial role in the normal progress of folliculogenesis. Using single and double GATA4 and GATA6 granulosa cell conditional knockout animals, we document that both factors are necessary for normal folliculogenesis and female fertility. Our findings confirmed that deletion of GATA4 leads to subfertility (88) and demonstrate that animals lacking GATA6 in granulosa cells have normal ovarian function. These results support our initial

hypothesis, which suggests that silencing both factors leads to a stronger ovarian phenotype when compared with the single knockouts.

The lack of an abnormal phenotype in GATA6 mutants may be due to a functional compensation by GATA4. In fact, the DNA-binding domains of mouse GATA4 and GATA6 proteins are 85% identical, allowing these factors to recognize similar DNA sequences (55-57). This characteristic may also explain the functional redundancy between GATA4 and GATA6 found in liver bud formation (99) and cardiac myocyte differentiation (100). Overlapping functions for GATA4 and GATA6 have also been described in the jejunum and duodenum where conditional deletion of GATA6 has no effect; however, a strong phenotype was observed when both GATA6 and GATA4 were deleted (101). Interestingly, similar to our findings, conditional deletion of GATA4 in the jejunum has profound effects even in the presence of GATA6 (102). Taken together, this evidence suggests that GATA4 can regulate specific GATA6 gene targets. Considering that double-knockout animals are infertile whereas single GATA4 knockout are subfertile, it is also possible to conclude that GATA6 is able to compensate, at least partially, for the lack of GATA4. This finding also suggests that GATA6 may play a crucial role in ovarian function. In the next chapter, I explored the possible mechanisms involved in the full or partial compensation observed between GATA4 and GATA6.

Lack of ovulation due to an ovarian defect seems to be the major cause of infertility in G4/6^{gcko} mice because these animals have elevated levels of FSH and eCG treatment does not stimulate the formation of preovulatory follicles. In the ovaries of nontreated double-knockout animals, however, small antral follicles were regularly found. These puzzling differences can be attributed to a much stronger and earlier activation of the Cyp19a1 promoter by eCG, as previously demonstrated (81). It is possible that in eCG-treated mice the silencing of GATA factors occurs before the formation of antral follicles, leading to a complete halt in follicle growth. Granulosa differentiation to the preovulatory stage is impaired in G4^{gcko} and G4/6^{gcko} females. The low expression of aromatase, a classical marker of granulosa cell differentiation, supports this conclusion and suggests an attenuated response to FSH. In

fact, our findings demonstrate that in the absence of GATA4 or GATA4 and GATA6, the FSHR protein was undetectable by Western blot. We also demonstrated that GATA4, by interacting with a GATA-RE, increases the activity of the *Fshr* promoter. FSH is crucial for follicle growth and granulosa cell differentiation, as pointed out by the phenotype observed in FSHR (13) and FSHβ-subunit (12) knockout mice. FSHR- and FSH-deficient females are infertile due to a block in folliculogenesis before antral follicle formation. This block in folliculogenesis also occurs in the double GATA4/6^{gcko} animals treated with eCG, in which follicles do not grow beyond the multilayered preantral stage. At this point in follicular development, FSH responsiveness is essential for the formation of the antrum and growth to the preovulatory stage (103). Therefore, the down-regulation of the FSHR in the granulosa cells provides a molecular mechanism that explains the abnormal follicle growth and infertility of GATA conditional knockout animals.

Collectively, these data provide strong evidence that GATA4 and GATA6 have overlapping but essential roles in the ovary and both are needed to ensure proper follicle growth, granulosa cell differentiation, and female fertility. Additionally, our *in vivo* and *in vitro* findings are consistent with a role for GATA4 in the regulation of FSHR expression in the ovary. Considering that FSH signaling is crucial for normal folliculogenesis, GATA4 stimulation of the FSHR may be crucial for normal follicle development, granulosa cell differentiation, and, ultimately, female fertility. This evidence provides a likely molecular mechanism to explain the subfertility of single G4^{gcko} mice (88) and the infertility phenotype of the double GATA4 and GATA6 conditional knockout mice.

IV. GATA4 AND GATA6 SILENCING IN OVARIAN GRANULOSA CELLS AFFECTS MESSANGER RNA LEVELS IN GENES INVOLVED IN STEROIDOGENESIS, EXTRACELLULAR STRUCTURE ORGANIZATION, IGF1 ACTIVITY AND APOPTOSIS

A. Introduction

As it was shown in the previous chapter, the transcription factors GATA4 and GATA6 are crucial for normal granulosa cell differentiation (83). In the mouse, the GATA4 and GATA6 genes encode proteins of 48 and 45 kDa, respectively, which are 85% identical at the amino acid level within the DNA binding region (57). Consequently, both GATA factors recognize a conserved binding motif characterized by the core A/T-GATA-A/G (104). This property of GATA4 and GATA6 impedes determination of the genes and functional pathways targeted by each factor in the ovary. On the other hand, this particularity of GATA4 and GATA6 could account for the functional compensations observed when one or the other is silenced in granulosa cells (GCs). For instance, as shown in chapter III, $G4^{gcko}$ mice are sub-fertile whereas $G6^{gcko}$ mice have no reproductive defects; in marked contrast, animals lacking both GATA4 and GATA6 in GCs are infertile (83). The mechanisms responsible for the redundant or compensatory roles of GATA4 and GATA6 in the ovary are not fully understood.

These findings also indicate that GATA4 and GATA6 do not contribute equally to regulate ovarian function and that GATA4 plays a major role in the regulation of follicle growth and maturation. Thus, mice lacking GATA4 in GCs release significantly fewer oocytes at ovulation than wildtype animals (83,88); however, mice lacking GATA6 ovulate normally. In addition, GATA4, but not GATA6, binds to the promoters of the aromatase (Cyp19a1) and the follicle stimulating hormone receptor (FSHR) genes (71,83), which are essential for normal follicle growth. Moreover, we have previously shown a significant decrease in the expression of Cyp19a1, Lhcgr, Cyp11a1, and FSHR only in the absence of GATA4 (Chapter III). Interestingly, although genes targeted specifically by GATA6 have not been described in GCs, GATA6 compensates for the absence of GATA4 and partially sustains GC function (83), suggesting that GATA6 is able to replace GATA4 in the stimulation of key genes involved in folliculogenesis. The identity of these genes remains unknown.

In this chapter, we examined the impact that the lack of GATA4, GATA6 or both has on genome-wide gene expression during the process of GC differentiation. Elucidation of the genes regulated by GATA4 and/or GATA6 is essential to provide novel insights into the transcriptional regulatory programs controlled by each factor. The results of this analysis revealed a role for GATA factors in the regulation of genes involved in ovulation, steroid metabolic processes, extracellular structure organization, insulin-like growth factor metabolism, and intracellular signaling.

B. Results and Discussion

1. Genes Regulated by Silencing of GATA

The knockdown of GATA4 and GATA6 in GCs impairs folliculogenesis and causes female infertility (83). In an effort to uncover the transcriptional defects that lead to these phenotypes, we performed mRNA microarray analyses on GCs of wildtype (WT), $G4^{gcko}$, $G6^{gcko}$, or $G4/6^{gcko}$, animals treated with eCG for 48 hours. Analysis of differentially expressed genes between the GATA conditional knockouts and WT demonstrated that more genes were affected by the absence of both GATA4 and GATA6 than in the absence of either factor alone (Fig 15A-C). Microarray data were analyzed using Significance Analysis of Microarrays (SAM) (false discovery rate: 0.01, 1000 permutations, confidence level: 90%) (105). One-way ANOVA analysis of three independent samples for each genotype was used to identify genes in which expression changed by twofold or more between knockouts and WT. This analysis revealed that 493 genes in $G4/6^{gcko}$, 224 genes in $G4^{gcko}$, and 34 genes in $G6^{gcko}$ were significantly regulated by twofold or more ($P<0.01$). A list of all differentially expressed genes can be found in Tables III ($G4^{gcko}$), IV ($G6^{gcko}$), and V ($G4/6^{gcko}$) (Appendix A).

The overlap of differentially regulated genes between the three phenotypes was represented using a Venn diagram (Fig.15D). This diagram revealed a greater degree of overlap between $G4^{gcko}$ and $G4/6^{gcko}$ than between $G6^{gcko}$ and $G4/6^{gcko}$ or between $G6^{gcko}$ and $G4^{gcko}$ (Fig. 15D). Venn diagrams of downregulated or upregulated genes yielded similar findings (Fig. 15E and 15F). Genes regulated by

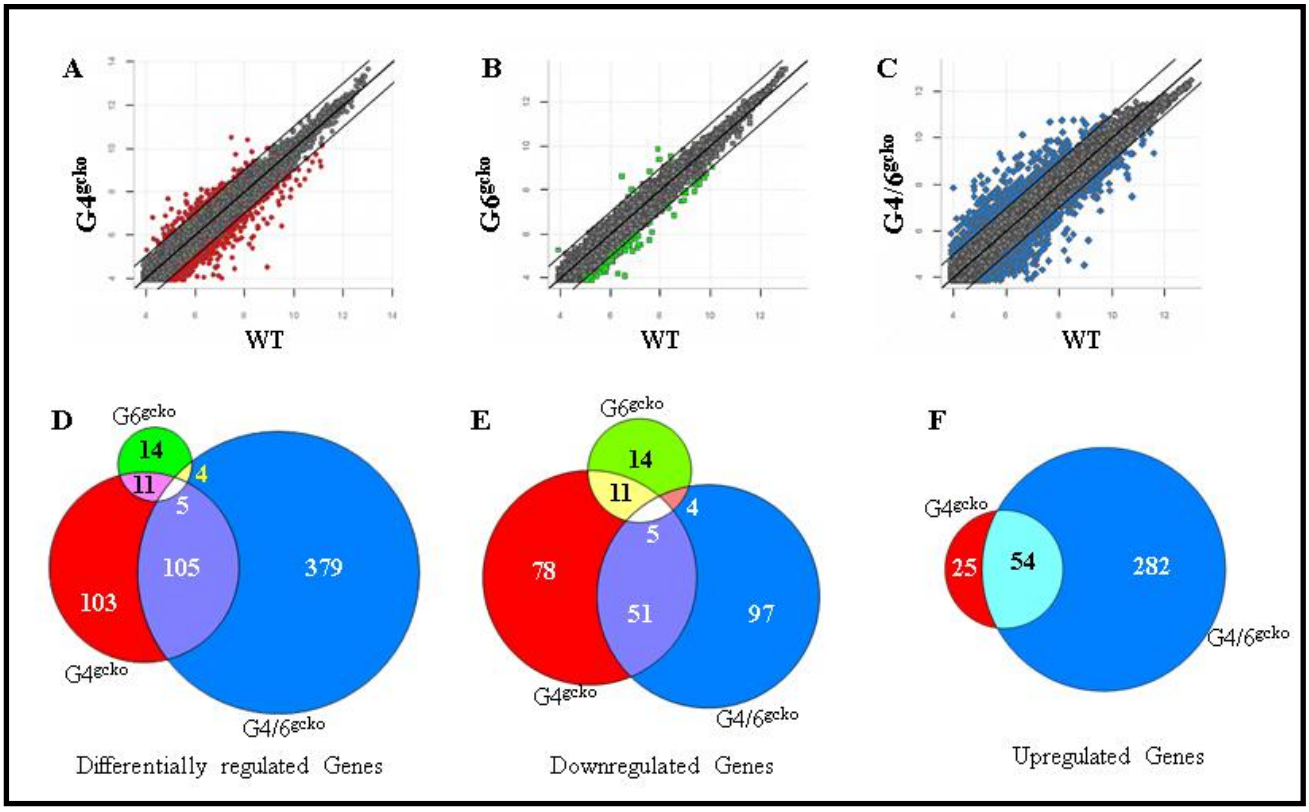


Figure 15. Gene Expression Profiles in Wildtype (WT), GATA4, GATA6, and GATA4/6 Conditional Knockout Animals

A-C: Scatter plot of gene expression profiles of GATA knockouts versus wildtype (WT) ovarian granulosa cells. Each *point* represents a unique probe set. Y- and X-axis values are expressed as the logarithm of expression intensity for each probe set. The middle diagonal line represents equal expression. Probe sets that yielded a twofold difference (as determined by SAM analysis) are located outside (up or down) of the outlier lines that indicate ± twofold between the mean of the ratios. **D-F**: Venn diagrams of gene expression profiles for each genotype. *Numbers* indicate total mRNAs significantly regulated in common between GATA4, GATA6, and GATA4/6 as well as those individually regulated by each genotype. Lists of the genes included in each one of these categories can be found in Tables III-VI (Appendix A).

each phenotype as well as the elements that are common between genotypes are listed in Tables VIA-D (Appendix A). These findings confirm the predominant role of GATA4 in the regulation of GC function and provided for the first time a short list of genes that seem to be exclusive targets of GATA6. Based on these findings, it is also possible to conclude that GATA4 and GATA6 compensate for one another in the regulation of GC function.

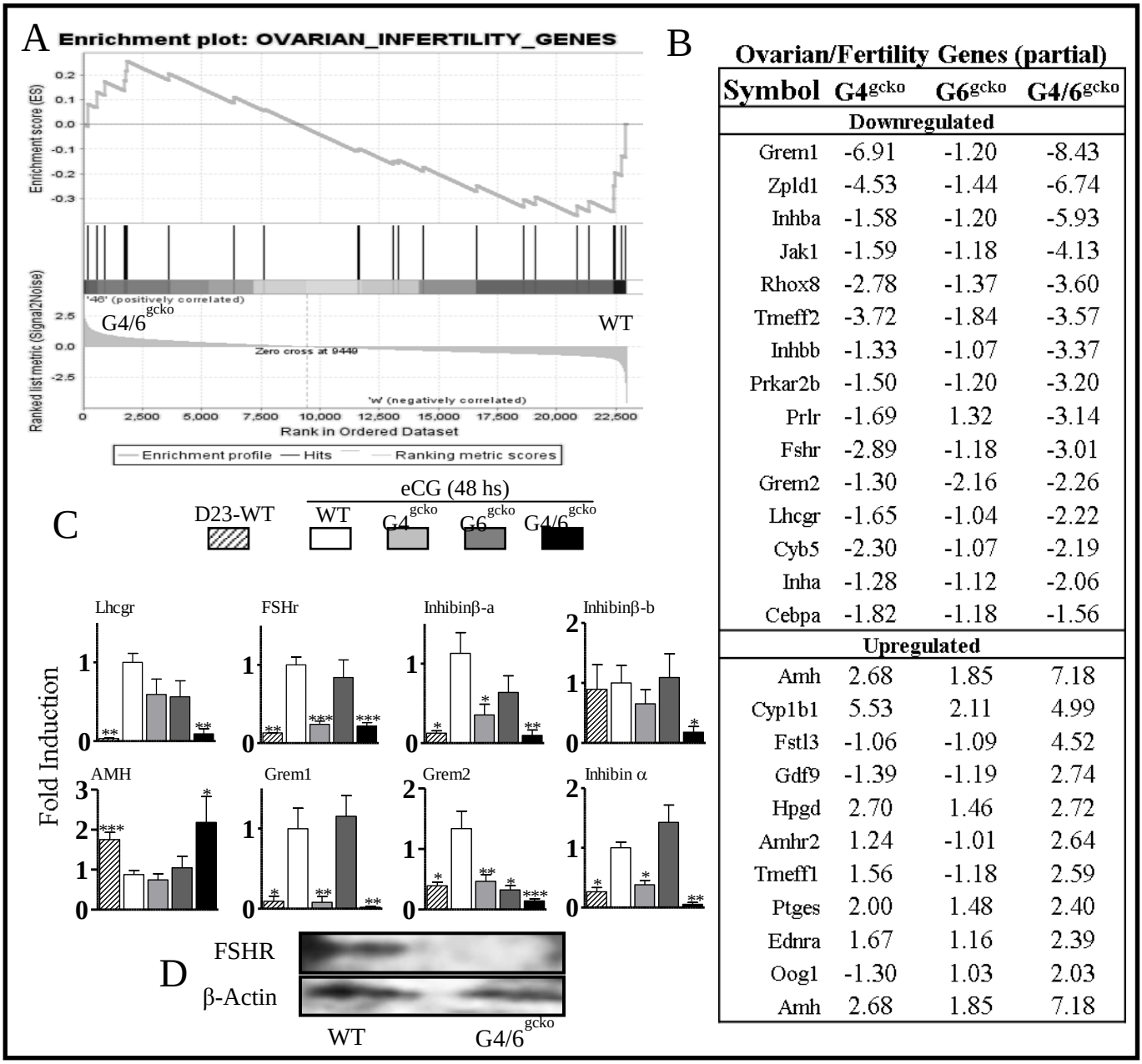
2. Functional Classification of GATA-regulated Genes

In view of the compensatory actions observed between GATA4 and GATA6, we next performed functional analyses of genes significantly ($P<0.01$) regulated in the absence of both GATA4 and GATA6 using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/), Significance Analysis of Microarrays (SAM), and Gene Set Enrichment Analysis (GSEA) (106,107). Multiple pathways, including ovulation-related genes, ovarian/infertility genes, steroid metabolic process, extracellular organization, regulation of growth, and intracellular signaling were found to be affected (Table VII, Appendix A).

3. Ovulation-related and Ovarian/Infertility Genes

GSEA analyses identified genes associated with ovarian defects and infertility to be significantly represented within the differentially regulated genes in G4/6^{gcko} GCs (Fig. 16A). A partial list of these genes is shown in Fig. 16B. Of these genes, inhibin βa and βb subunits (Inhβa and Inhβb), inhibin-α (Inhα), FSHR, Lhcgr, gremlin 1 and 2 (Grem1 and 2), peroxisome proliferator activated receptor gamma (Pparg), CCAAT/enhancer binding protein alpha (Cebpa), and prolactin receptor (Prlr) were downregulated. Whereas genes such as endothelin receptor type A (Ednra), follistatin-like 3 (Fstl3), cytochrome P450, family 1, subfamily b, polypeptide 1 (Cyp1b1), and anti-Mullerian hormone (Amh) were upregulated.

To confirm the microarray results, we performed new experiments in which immature wildtype or GATA conditional knockout mice were treated with eCG to stimulate follicle maturation and GC differentiation. As a control, 23-day old unstimulated females (D23) were also included. The expression



Ovarian/Fertility Genes (partial)

Symbol	G4^{gcko}	G6^{gcko}	G4/6^{gcko}
Downregulated			
Grem1	-6.91	-1.20	-8.43
Zpld1	-4.53	-1.44	-6.74
Inhba	-1.58	-1.20	-5.93
Jak1	-1.59	-1.18	-4.13
Rhox8	-2.78	-1.37	-3.60
Tmeff2	-3.72	-1.84	-3.57
Inhbb	-1.33	-1.07	-3.37
Prkar2b	-1.50	-1.20	-3.20
Prlr	-1.69	1.32	-3.14
Fshr	-2.89	-1.18	-3.01
Grem2	-1.30	-2.16	-2.26
Lhcgr	-1.65	-1.04	-2.22
Cyb5	-2.30	-1.07	-2.19
Inha	-1.28	-1.12	-2.06
Cebpa	-1.82	-1.18	-1.56
Upregulated			
Amh	2.68	1.85	7.18
Cyp1b1	5.53	2.11	4.99
Fstl3	-1.06	-1.09	4.52
Gdf9	-1.39	-1.19	2.74
Hpgd	2.70	1.46	2.72
Amhr2	1.24	-1.01	2.64
Tmeff1	1.56	-1.18	2.59
Ptges	2.00	1.48	2.40
Ednra	1.67	1.16	2.39
Oog1	-1.30	1.03	2.03
Amh	2.68	1.85	7.18

Figure 16. Ovarian and Fertility Related Genes

A: GSEA enrichment plot for ovulatory and infertility genes. **B**: List of selected ovulatory/fertility genes from the GSEA and DAVID analysis. **C**: qPCR determination of selected differentially regulated genes in untreated D23 WT animals and in eCG-treated WT, GATA4, GATA6, and GATA4/6 conditional knockout animals. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM (*, $P< 0.05$; **, $P< 0.01$; ***: $P< 0.001$ vs. WT one-way ANOVA, Tukey test). D: FSH receptor protein (FSHR) levels in granulosa cells from D23 eCG-treated WT and G4/6^{gcko} animals. β-actin was used as a loading control.

of selected genes was determined using qPCR (Fig. 16C). Confirming our previous report (83) and microarray results (Fig. 16B), qPCR assays demonstrated the essential role of GATA4 in the regulation of FSHR and Lhcgr expression (Fig. 16C). In addition, western blot analyses demonstrated that FSHR protein expression is undetectable in GCs of G4/6^{gcko} animals (Fig. 16D).

Inhibins are heterodimers of the common α subunit with either βa or βb subunits (inhibins A and B respectively). The expression of the α subunit is regulated by GATA factors *in vitro* (108,109). Our findings demonstrated that GATA factors are also required for the expression of the α subunit *in vivo*. Thus, microarray results revealed a twofold decrease in the expression of the α subunit in G4/6^{gcko} when compared with wildtype animals. This finding was confirmed by qPCR showing that the lack of GATA4 or GATA4 and GATA6 decreased α subunit expression to levels observed in untreated D23 animals (Fig. 16C). We also provided evidence that GATA4 and GATA6 participate in the regulation of the two β subunits. Thus, in eCG-treated animals, microarray and qPCR data demonstrated a decrease in the mRNA expression of the βa (sixfold) and βb (threefold) subunits in the absence of GATA4 and GATA6 when compared with WT animals treated with eCG (Fig. 16B and 16C). Moreover, qPCR data demonstrated that the expression of the βa and α subunits, but not that of βb subunit, is stimulated by eCG (Fig. 16C). The ratio of inhibin/activin changes as follicles grow (66); thus, pre-antral follicles produce mainly activins (ββ dimer), whereas preovulatory follicles produce mostly inhibin A (βa/α dimer) (110). Our *in vivo* results suggest that GATA factors are required for the increase in inhibin A observed in pre-ovulatory follicles.

In contrast to the inhibins, anti-müllerian hormone (AMH) is highly expressed in GCs of pre-antral and early antral follicles (111) and progressively decreases toward the preovulatory stage (104,112,113). In agreement with the lack of follicle maturation observed in the absence of GATA factors (83), AMH mRNA expression was higher in the ovary of G4/6^{gcko} animals when compared with WT (Fig. 16B and 16C). These are intriguing findings since the expression of AMH has been shown to be

stimulated by GATA4 (55). The mechanism that leads to the sustained expression of AMH in GATA4 deficient GCs needs further analysis.

Microarray analysis also revealed that the lack of GATA4 and GATA6 affects the expression of Grem1 and Grem2 in GCs. Grem1 was significantly lower in $G4^{gcko}$ and $G4/6^{gcko}$ animals. In contrast, Grem2 expression decreased by twofold in the $G6^{gcko}$ and in $G4/6^{gcko}$ animals (Fig. 16B) suggesting that GATA4 and GATA6 may specifically target Grem1 and Grem2, respectively. Grem1 expression in GCs is stimulated by eCG (114,115) and by GDF9 and bone morphogenetic protein 4 (115). Both Grem1 and Grem2 prevent the inhibitory effect of BMP4 on granulosa cell steroidogenesis (114). Our findings demonstrate that the absence of GATA factors abolished the increase of Grem1 and Grem2 expression induced by eCG. Therefore, GATA4 and GATA6 may contribute to the normal development of folliculogenesis by mediating the effects of GDF9 on Grem1/2 expression. Noteworthy, Grem2 is one of the few genes regulated in the absence of GATA6. However, qPCR analysis indicated that the expression Grem2 was low in the absence of either GATA factor; although, Grem2 expression tended to be lower in the absence of GATA6 than in the absence of GATA4. Whether the Grem2 gene can be specifically regulated by GATA6 remains to be determined.

4. <u>Steroid Synthesis</u>

Several genes involved in steroid metabolism were differentially regulated in GATA conditional knockout animals (Fig. 17A and 17B). Steroidogenic genes including Akr1c18 (progesterone metabolism), CYP11a1 (progesterone synthesis), and Cyp19a1 (estrogen synthesis) were decreased in eCG-treated $G4/6^{gcko}$ mice by ninefold, twofold, or 11-fold respectively when compared with eCG-treated WT animals. A downregulation of Cyp19a1, aldo-keto reductase family 1, member C18 (Akr1c18), and Cyp11a1 expression was also seen in the $G4^{gcko}$ animals. Ferredoxin 1 (Fdx1), which shuttles electrons from ferredoxin reductase to Cyp11a1, was significantly decreased in $G4/6^{gcko}$ when compared with wildtype and single knockout animals treated with eCG. Additionally, eCG stimulation of low-density lipoprotein receptor (Ldlr), which is required for the uptake of cholesterol needed for steroid synthesis, was significantly decreased in $G4/6^{gcko}$ animals (Fig. 17A and 17B). Cytochrome

A

Steroidogenic Genes

Symbol	G4^{gcko}	G6^{gcko}	G4/6^{gcko}
Downregulated			
Cyp19a1	-3.55	-1.46	-11.58
Akr1c18	-7.44	-2.27	-8.78
Fdx1	-2.14	1.15	-1.90
Pparg	-1.11	-1.22	-2.57
Ldlr	-1.45	1.14	-1.96
Hmgcr	-1.54	1.07	-1.91
Cyb5	-2.30	-1.07	-2.19
Cyp11a1	-2.32	-1.36	-2.09
Upregulated			
Apoe	2.06	1.66	6.26
Cyp1b1	5.53	2.11	4.99
StAR	-1.62	1.52	1.50

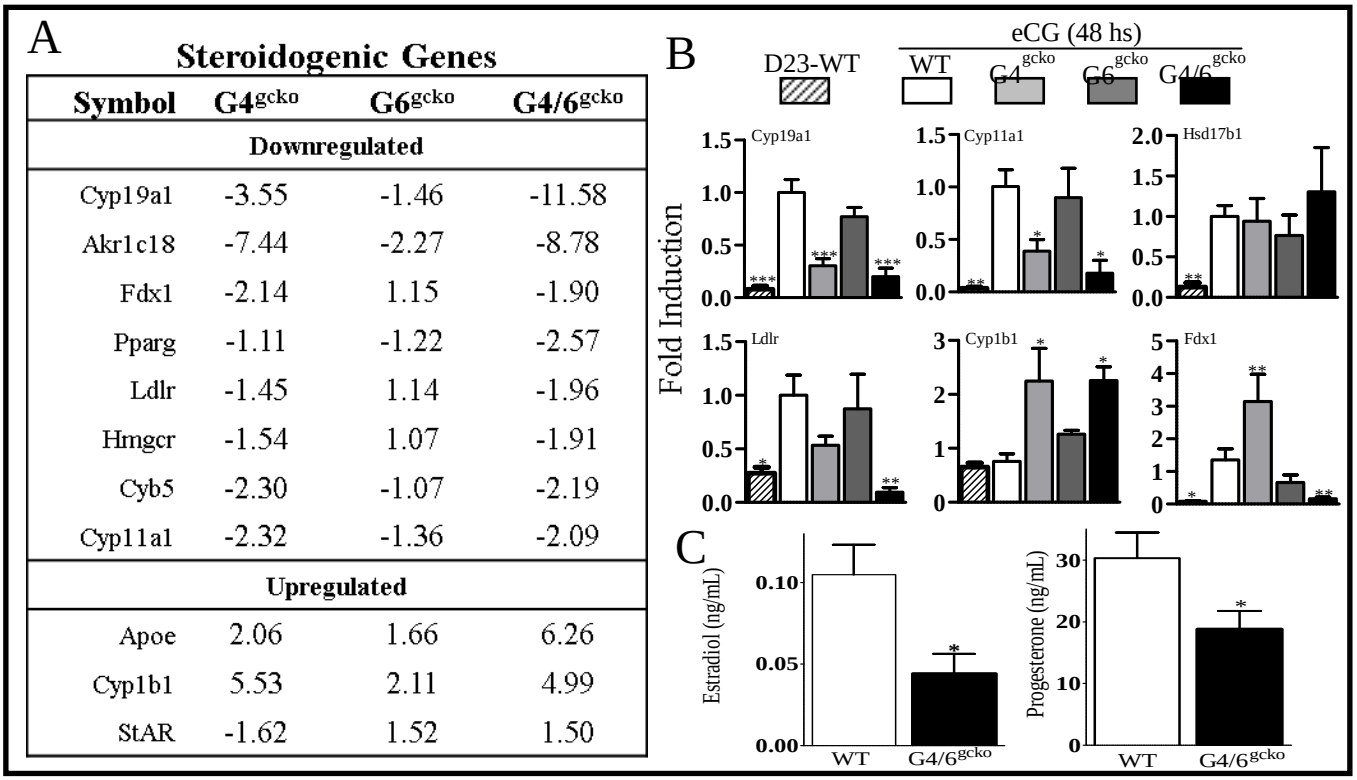


Figure 17. Steroidogenic Genes

A: List of selected steroidogenic genes from DAVID and GSEA analysis of differentially regulated genes. **B**: Relative expression of steroidogenic genes in untreated D23 WT animals and in eCG-treated WT, GATA4, GATA6, and GATA4/6 conditional knockout animals. **C:** Progesterone and estradiol levels after 48 hs treatment with eCG of WT or G4/6^{gcko} animals. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. WT one-way ANOVA, Tukey test).

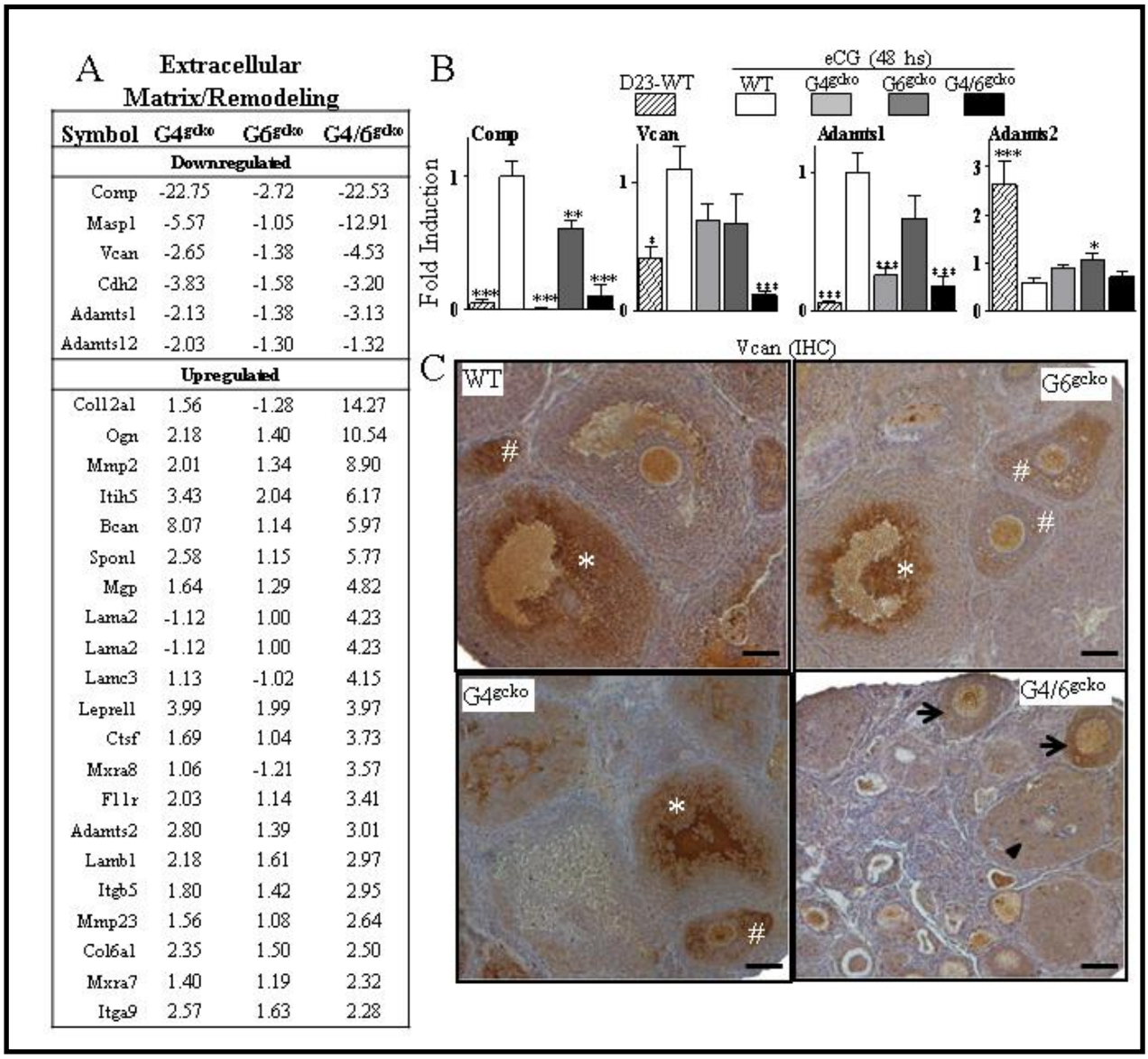
P4501b1 (Cyp1b1) that inactivates estradiol (116) increased by fivefold in $G4^{gcko}$ and $G4/6^{gcko}$ animals. These findings suggest that, in the absence of GATA4 and GATA6 expression, the synthesis of estradiol and progesterone is significantly impaired. This conclusion is supported by the decrease in estradiol and progesterone serum levels observed in $G4/6^{gcko}$ when compared with WT animals (Fig. 17C).

A sixfold increase in apolipoprotein-E (Apoe) was observed in $G4/6^{gcko}$ animals. In the ovary, Apoe may limit androgen production, thereby limiting follicular estrogen synthesis. In fact, in humans the levels of Apoe in follicular fluid decrease as serum estrogen levels increase during the menstrual cycle (117). In addition, Apoe has been shown to increase in atretic follicles (118), which correspond with the increase in apoptosis observed in follicles lacking GATA factors (see below).

Taken together these findings suggest that the increased expression of Cy1pb1 and Apoe along with a decrease in aromatase expression may contribute to the decrease in estradiol levels observed in and $G4/6^{gcko}$ animals (Fig. 17C).

5. <u>Extracellular Structure Organization</u>

DAVID analysis of differentially expressed genes revealed an enrichment of genes involved in the reorganization of the extracellular matrix (ECM) and cell adhesion (Table VII, Appendix A). Similarly, GSEA analysis identified a set of genes associated with ECM proteins (not shown). A list of selected genes identified by these two analyses is shown in Fig. 18A. Within these genes, ADAMTS1 and versican (Vcan) are known to be crucial for normal ovulation (41,119). ADAMTS (A Disintegrin and Metalloproteinase with Thrombo Spondin motifs) are proteinases that cleave proteoglycans present in the ECM that surrounds all cells and tissues. Of the proteoglycans present in the ovarian ECM, Vcan is produced by GCs and can be found in the granulosa layer of small growing follicles and in antral follicles (120,121). These findings suggest that Vcan is a matrix component of the follicle expressed throughout folliculogenesis. Data from the microarray and qPCR experiments suggest that the expression of ADAMTS1 and Vcan increases after treatment of WT animals with eCG. However, this increase in the expression of ADAMTS1 and Vcan was abolished by the deletion of both GATA4 and



Symbol	G4^{gcko}	G6^{gcko}	G4/6^{gcko}
Downregulated			
Comp	-22.75	-2.72	-22.53
Masp1	-5.57	-1.05	-12.91
Vcan	-2.65	-1.38	-4.53
Cdh2	-3.83	-1.58	-3.20
Adamts1	-2.13	-1.38	-3.13
Adamts12	-2.03	-1.30	-1.32
Upregulated			
Col12a1	1.56	-1.28	14.27
Ogn	2.18	1.40	10.54
Mmp2	2.01	1.34	8.90
Itih5	3.43	2.04	6.17
Bcan	8.07	1.14	5.97
Sponl	2.58	1.15	5.77
Mgp	1.64	1.29	4.82
Lama2	-1.12	1.00	4.23
Lama2	-1.12	1.00	4.23
Lamc3	1.13	-1.02	4.15
Leprell	3.99	1.99	3.97
Ctsf	1.69	1.04	3.73
Mxra8	1.06	-1.21	3.57
F11r	2.03	1.14	3.41
Adamts2	2.80	1.39	3.01
Lamb1	2.18	1.61	2.97
Itgb5	1.80	1.42	2.95
Mmp23	1.56	1.08	2.64
Col6a1	2.35	1.50	2.50
Mxra7	1.40	1.19	2.32
Itga9	2.57	1.63	2.28

Figure 18. Extracellular Matrix and Tissue Remodeling Genes.

A: List of selected differentially regulated genes involved in extracellular matrix/structural remodeling as determined by DAVID analysis. **B**: qPCR quantification of key extracellular/remodeling genes in the different genetic backgrounds. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM. (*, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$ vs. WT one-way ANOVA, Tukey test). **C**: Immunohistochemical (IHC) analysis of versican (Vcan) in WT, G4^{gcko}, G6^{gcko} and G4/6^{gcko} ovaries from D23 eCG-stimulated mice. (n = 3 for each genotype; representative pictures are shown). #: Secondary follicles; *: antral follicles; ➔: secondary follicles; ▶: early antral follicles. Versican staining is depicted in brown, counterstaining by hematoxylin is depicted in light blue.

GATA6 (Fig. 18B). Thus, after treatment with eCG, the expression of ADAMTS1 and Vcan was fivefold and 10-fold lower respectively in G4/6^{gcko} when compared with WT (Fig. 18B). This decrease in the expression of Vcan was confirmed by IHC staining with an antibody that detects the V0 and V1 isoforms of Vcan. There was diffuse Vcan staining in the granulosa layer of antral follicles, but very intense staining within follicular antrum and cumulus cells (Fig. 18C). Similarly, Vcan was detected in antral and secondary follicles of WT, G4^{gcko} and G6^{gcko} animals. In contrast, in G4/6^{gcko} animals, Vcan was detectable in early secondary follicles (Fig. 18C arrows), but not in any of the few early antral follicles that these animals develop (Fig. 18C arrowhead). This pattern of Vcan expression was also observed at the RNA level (Fig. 18B).

Cartilage oligomeric matrix protein (Comp) mRNA levels were significantly decreased (22-fold) in G4^{gcko} and G4/6^{gcko} mice when compared with WT mice. In the absence of GATA6 a twofold decrease in Comp expression was also observed (Fig. 18A). qPCR analysis confirmed the downregulation of Comp in G4^{gcko}, G6^{gcko} and G4/6^{gcko} animals (Fig. 18B) and demonstrated that eCG treatment induces a 20-fold stimulation of Comp expression. The role that Comp may play in the regulation of follicle development remains to be determined.

In contrast to the dramatic decrease in the expression of Comp, ADAMTS1, and Vcan observed in conditional knockout animals, we observed an increase in the expression of some ECM related proteins and enzymes. For instance, the expression of Collagen 12 and 6, Laminin a1/2, b1 and c3, and several integrins increased in the absence of GATA factors. Similarly, the expression of ADAMTS2 increased in GATA4^{gcko} and GATA4/6^{gcko} animals.

Taken together these findings suggest that in the absence of GATA factors the remodeling of the follicular ECM is greatly compromised. One important step in the process of follicle maturation is the formation of the antrum. Antrum formation does not occur in G4/6^{gcko} animals, suggesting that GATA4 and GATA6 are needed for the build-up of the extracellular components involved in this process.

6. Insulin-like Growth Factor (IGF) 1 and IGF Binding Protein (IGFBP) System

DAVID analysis indicated that the lack of GATA4 and GATA6 expression in GCs negatively affects the biological activity of IGF1 ($P<6.8x10^{-5}$). IGF1 is required for the differentiation of GCs to the preovulatory stage (122). Although a small 1.7-fold reduction of IGF1 expression was observed, microarray results demonstrated that IGF1 binding proteins (IGFBP) 2, 4, and 5 remained highly expressed in GCs lacking GATA factors (Fig. 19A). In particular, IGFBP4 was significantly upregulated in $G4^{gcko}$ (4.4-fold), $G6^{gcko}$ (1.93-fold), and $G4/6^{gcko}$ (21.92-fold) and was within the most upregulated genes in all three knockouts. IGFBPs inhibit the interaction of IGF1 with the IGF1 receptor and are known to prevent follicular growth and maturation (123,124). IGFBP expression has been shown to be downregulated during the differentiation of GCs to the preovulatory stage (125-127). qPCR assays confirmed the upregulation of IGFBP2 (3-fold), IGFBP4 (4-fold) and IGFBP5 (2-fold) in the absence of GATA4; however, in the absence of both GATA factors only IGFBP4 (11-fold) and IGFBP5 (4-fold) were upregulated (Fig. 19B).

IGFBP5 overexpressing female mice are subfertile (128), whereas IGFBP4 inhibits LH-induced progesterone and FSH-induced estradiol production in human GCs (129). IGFBP levels are mainly regulated by proteolytic degradation by specific proteinases such as pregnancy-associated plasma protein-A (Papp-a), which is highly expressed in healthy antral follicles and positively correlates with dominant follicle development (130,131). Accordingly, Papp-a knockout animals have a reduced number of pups per litter, a reduced number of oocytes ovulated and low estradiol levels after eCG stimulation (132,133). Papp-a expression in GCs was significantly decreased in GATA4 (4.24-fold) and GATA4/6 (-6.47), suggesting that GATA factors indirectly contribute to regulate IGF1 activity by regulating Papp-a expression

In addition, a twofold increase of PI3K Interacting Protein 1 (PIK3IP1), which binds to the p110 catalytic subunit of phosphatidylinositol-3-kinase (PI3K) and reduces its activity (68), was found in animals lacking GATA4 and GATA6. Since activation of PI3K is part of the canonical pathway

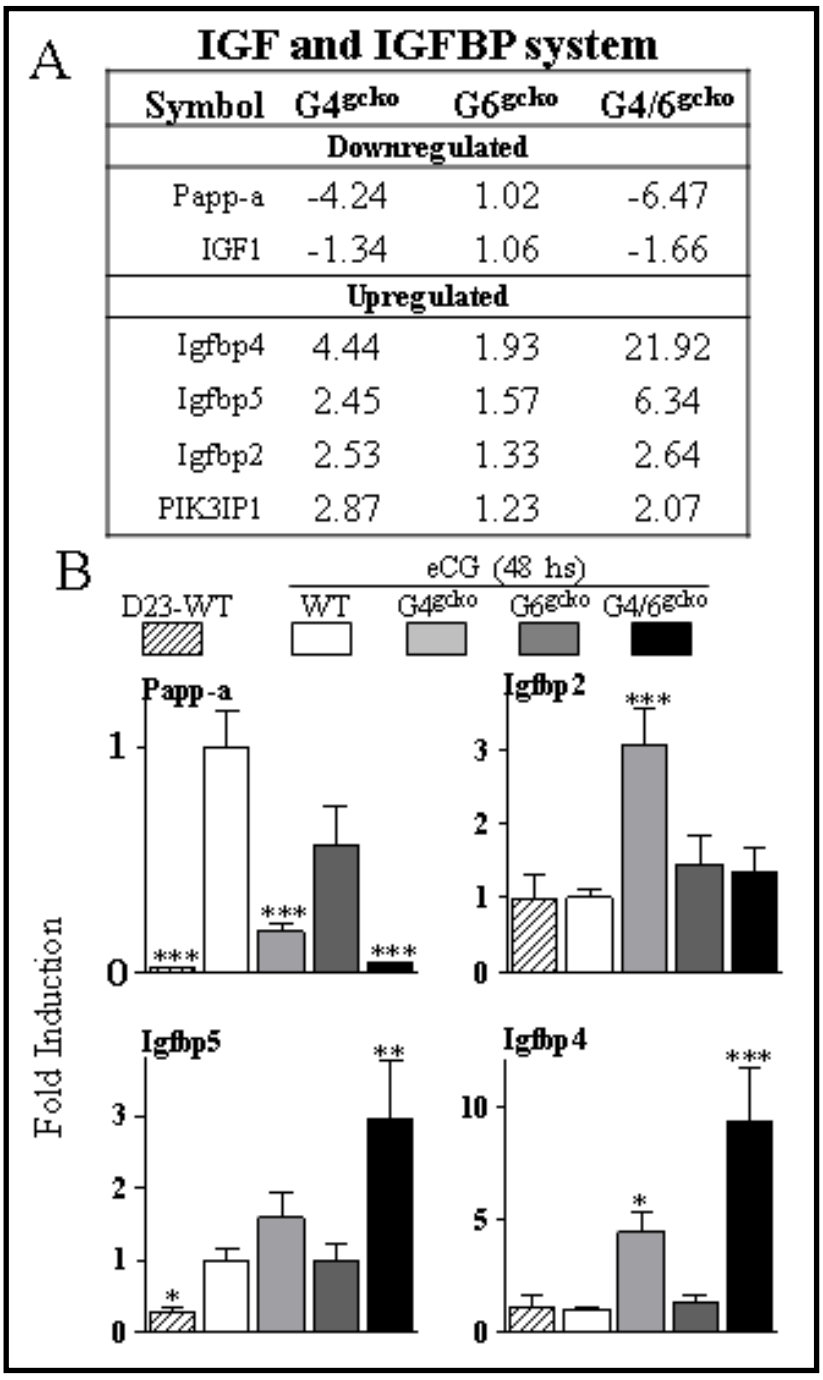


Figure 19. Insulin-like Growth Factor Related Genes.

A) Differentially regulated genes related to the IGF1 signaling pathway in GATA conditional knockout and wildtype animals. **B**) qPCR results of IGF1 signaling pathway genes in the different genetic backgrounds. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM. (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. WT one-way ANOVA, Tukey test).

activated by IGF1, the increase in PIK3IP1 may further contribute to a decrease in IGF1 signaling. These findings suggest that in the absence of GATA factors, IGF1 biological activity declines due to a reduction of IGF1, an increase in IGFBPs and PIK3IP1 expression, and a decrease in Papp-a expression. These effects could significantly contribute to the lack of follicle growth and the infertility observed in G4/6^{gcko} animals.

7. Apoptosis/Cell Division

Genes involved in apoptosis and cell proliferation were affected by the deletion of GATA factors in GCs (Fig. 20A). Within these genes, defender against apoptotic cell death (DAD1), a negative regulator of programmed cell death (134), was expressed at significantly lower levels in cells lacking GATA than in WT cells. In agreement with this finding, staining for cleaved caspase 3, a marker of apoptosis, increased in the ovaries of G4^{gcko} and G4/6^{gcko} animals treated with eCG in comparison to WT treated animals (Fig. 20B).

In addition, an increase in the expression of Gas (growth arrest-specific) 1 and 6 genes, which are both known to inhibit cell proliferation (135,136), was observed in G4/6^{gcko} mice. We also found an increase in the expression of growth arrest and DNA damage-inducible proteins: GADD45A, GADD45B, and GADD45G. Supporting a decrease in cell proliferation in the absence of GATA factors, FSH-induced stimulation of cell proliferation was significantly reduced in G4^{gcko} and G4/6^{gcko} cells (Fig. 20C). This finding was confirmed *in vivo* by a decrease in proliferating cell nuclear antigen (PCNA), a marker of proliferation, staining in G4/6^{gcko} when compared with WT animals (Fig. 20D).

DEP domain containing mTOR-interacting protein (Depdc6) is a component of both mTOR1 and 2 complexes and negatively regulates mTOR function (137). mTOR activity is a reliable indicator of cell growth (138,139). Microarray results showed that Depdc6 remains highly expressed in the absence of GATA4 and GATA6 when compared to WT cells. In addition, the homeodomain-interacting protein kinase 2 (HIPK2) was significantly reduced in G4^{gcko} and G4/6^{gcko}. HIPK2 is involved in the regulation of cell survival and proliferation (140). Accordingly, Hipk2 null mice show reduced cell proliferation

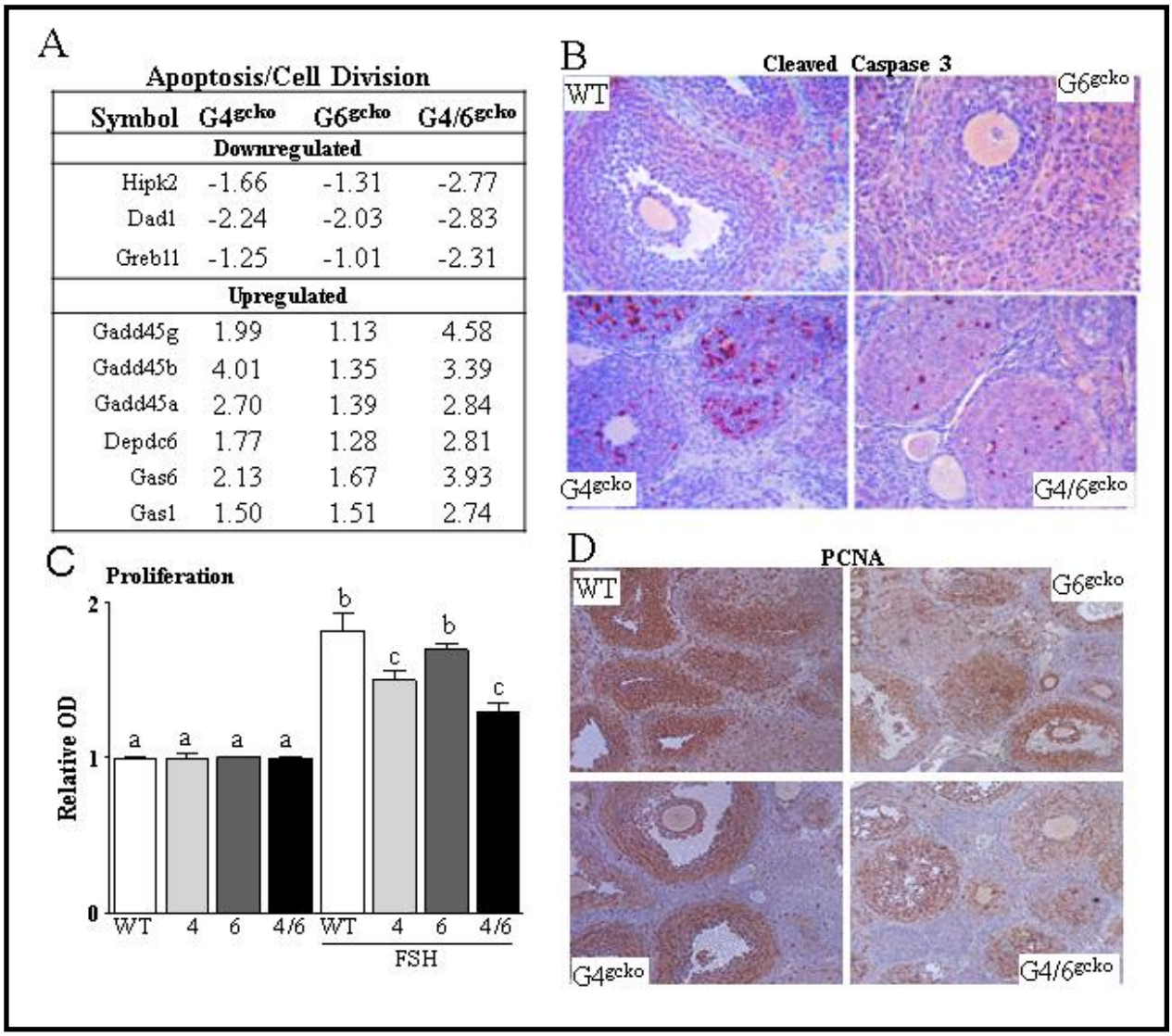


Figure 20. Cell Growth and Apoptosis Related Genes

A) List of selected genes involved in apoptosis and cell growth found to be significantly affected by the lack of GATA factor expression in granulosa cells. **B**) IHC for cleaved caspase 3 protein in WT, $G4^{gcko}$, $G6^{gcko}$ and $G4/6^{gcko}$ ovaries from D23 eCG-treated mice. (n = 3 for each genotype; representative pictures are shown). Cleaved caspase 3 staining is depicted in brown, counterstaining by hematoxylin is depicted in light blue. **C**) Proliferation, determined using MTT assays, of WT or GATA4/6 deficient granulosa cells. Proliferation was stimulated with 50 ng/mL of FSH. The experiment was repeated at least three times. *Columns* represent the mean ± SEM, columns with different letters differ significantly. **D**) IHC for PCNA in D23 eCG-treated WT or conditional knockout animals. (n = 3 for each genotype; representative pictures are shown). PCNA staining is depicted in brown, counterstaining by hematoxylin is depicted in light blue.

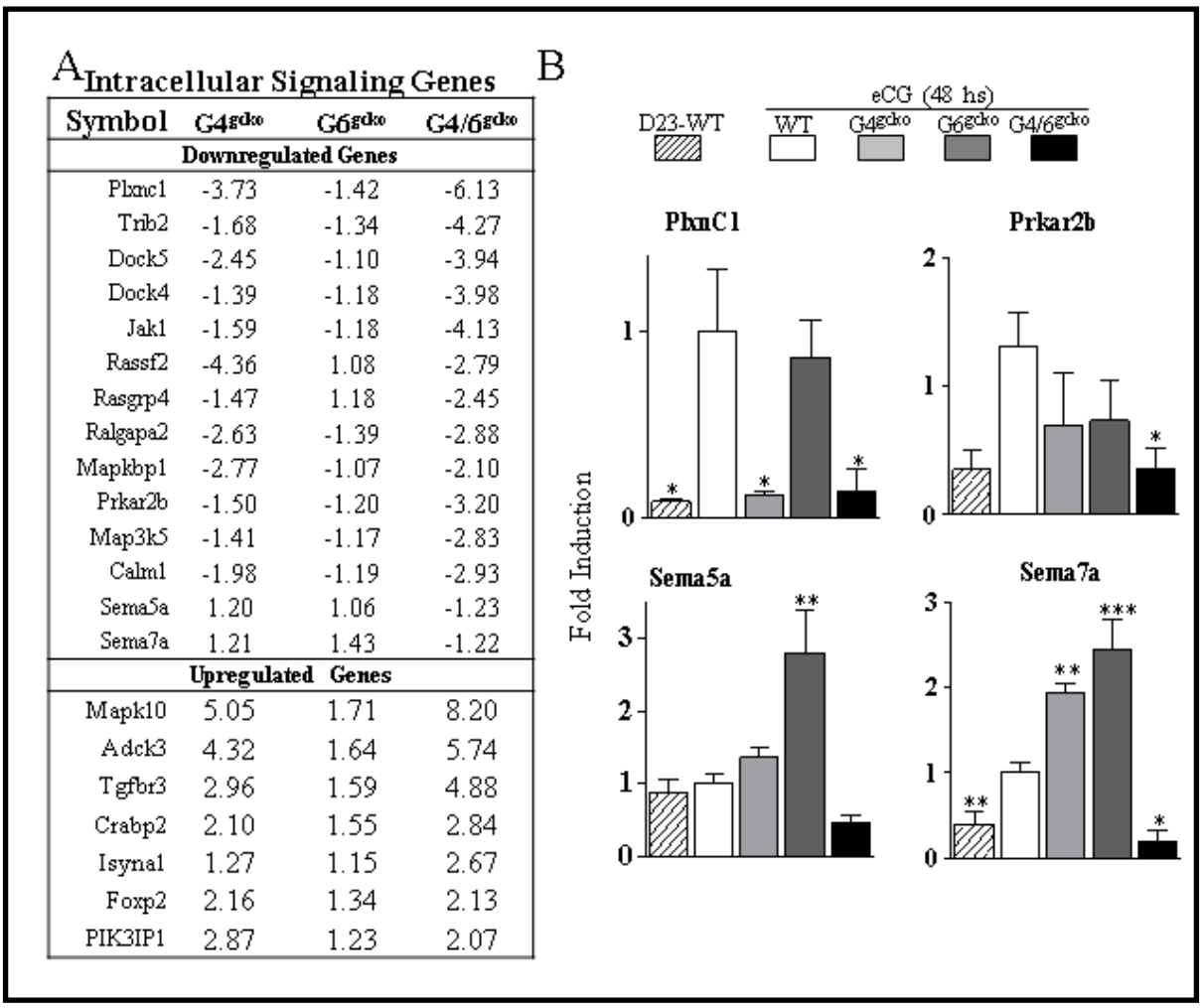
and accumulation of cells in the G0/G1 phase of the cell cycle (140). Thus, increased Depdc6 expression and decreased Hipk2 may contribute to the diminished amount of proliferation observed in conditional knockout cells.

These findings suggest that the lack of GATA factors in GCs not only increases apoptosis, but also halts GC proliferation. These effects are accomplished by an augmented expression of genes involved in growth arrest such as GAS1/6, GADD45a/b/g, and Depdc6 as well as decreased expression of proliferative factors such as HIPK2. To our knowledge, this is the first report suggesting a role for these genes in the proliferation of GCs.

8. Intracellular Signaling

DAVID and GSEA analyses showed an enrichment of genes involved in intracellular signaling. Of these genes, the expression of the protein kinase A (PKA) regulatory subunit R2b (Prkar2b) was significantly downregulated in the absence of GATA factors. PKA, which is crucial for normal ovarian function, is formed by two catalytic units and two regulatory (R) units of which four R subunits (R1a, R1b, R2a, and R2b) have been described (141). The expression of Prkar2b decreased by threefold in G4/6^{gcko} when compared with WT animals (Fig. 21A). Prkar2b is the most abundant R subunit expressed in GCs where it is stimulated by FSH (142,143). Our microarray analysis confirmed the abundance of R2b with respect to other units (ratio 2b/1a/2a/1b: 1/0.08/0.02/0.01); whereas qPCR results confirmed the stimulatory effect of FSH on the expression of Prkar2b (Fig. 21B). Moreover, these findings demonstrated that GATA4 and GATA6 are required for the stimulation of Prkar2b mRNA expression by FSH (Fig. 21B).

A sixfold to 10-fold decrease in the expression of the membrane receptor plexin C1 (PlxnC1) was observed in G4^{gcko} and G4/6^{gcko} animals (Fig. 21A and 21B). Plexins are receptors for semaphorins (Sema). PlexinB1 and its ligand Sema4D are expressed in ovarian follicles of mice under the control of FSH (144,145). We found that PlxnC1 and its ligand Sema7A are also highly expressed in GCs (Fig.



A

Intracellular Signaling Genes

Symbol	$G4^{gcko}$	$G6^{gcko}$	$G4/6^{gcko}$
Downregulated Genes			
Plxnc1	-3.73	-1.42	-6.13
Trib2	-1.68	-1.34	-4.27
Dock5	-2.45	-1.10	-3.94
Dock4	-1.39	-1.18	-3.98
Jak1	-1.59	-1.18	-4.13
Rassf2	-4.36	1.08	-2.79
Rasgrp4	-1.47	1.18	-2.45
Ralgapa2	-2.63	-1.39	-2.88
Mapkbp1	-2.77	-1.07	-2.10
Prkar2b	-1.50	-1.20	-3.20
Map3k5	-1.41	-1.17	-2.83
Calm1	-1.98	-1.19	-2.93
Sema5a	1.20	1.06	-1.23
Sema7a	1.21	1.43	-1.22
Upregulated Genes			
Mapk10	5.05	1.71	8.20
Adck3	4.32	1.64	5.74
Tgfbr3	2.96	1.59	4.88
Crabp2	2.10	1.55	2.84
Isyna1	1.27	1.15	2.67
Foxp2	2.16	1.34	2.13
PIK3IP1	2.87	1.23	2.07

Figure 21: Intracellular Signaling Related Genes.

A) Partial list of intracellular signaling related genes differentially regulated in conditional knockout versus WT animals. **B**) qPCR results for selected intracellular signaling genes in the different genetic backgrounds. Three or more animals were included for each genotype. *Columns* represent the mean ± SEM. (*, $P< 0.05$; **, $P< 0.01$; ***, $P< 0.001$ vs. WT one-way ANOVA, Tukey test).

21A and 21B). No significant changes in the expression of Sema7A were observed in the microarray analysis; however, by qPCR a fivefold decrease was observed in G4/6^{gcko} when compared with eCG-treated WT animals; whereas a significant increase in this mRNA was observed in both single knockouts (Fig. 19B). Although the function of these proteins in the ovary is unknown, PlxnC1 and Sema7A are involved in the regulation of cytoskeleton components including actin and cofilin (146,147). Cofilin inactivation plays an important role in ovarian steroidogenesis (148) suggesting that PlxnC1 may participate in the regulation of steroid synthesis in the ovary via regulation of cofilin.

The MAPK pathway has a crucial role in the regulation of folliculogenesis. For instance, disruption of Erk1/2 in mouse GCs impairs LH-induced oocyte resumption of meiosis, ovulation, and luteinization (149). The lack of GATA factors affected several members of the MAPK signaling pathways including Mapkbp1, Map3k5, and Mapk10. In addition, the expression of Rho and Ras small GTPases including Rhobtb1, Rnd2, Rassf2, Rasgrp4 and Ralgapa2 was affected in conditional knockout animals. In particular, expression of Ras was inhibited in the G4/6^{gcko}. Ras, via activation of the MAPK pathway, promotes growth, proliferation, differentiation and survival of cells (150). These findings suggest that a decrease in the expression of Ras signaling may lead to the reduction in proliferation observed in GCs lacking GATA factors.

9. Assessment of Gene Regulation by GATA Versus FSH Signaling Pathway

The results presented above demonstrated that one of the most important targets of GATA factors in GCs is the FSHR. Because of the central role of FSH in follicle maturation and GC differentiation, we further examined whether the changes in gene expression observed in GCs lacking GATA factors could be attributed entirely or in part to the decrease in FSHR expression. For this purpose, we isolated GCs from wildtype or GATA4$^{F/F}$:GATA6$^{F/F}$ immature animals. To induce the recombination of the floxed alleles, cells were infected with an adenovirus encoding CRE-recombinase (adCre). As expected, adCre reduced GATA4 and GATA6 expression by 98 and 97 percent,

respectively, in GCs containing floxed genes, but not in wildtype cells. Similarly, silencing of GATA factors resulted in the downregulation of the FSHR (not shown).

Next, we assessed the response of WT or GATA4/6-deficient GCs to cAMP, IGF1 or their combination. This approach bypassed the decrease of FSH and IGF1 signaling observed in cells lacking GATA factors. The results of these experiments suggested the presence of three groups of genes based on whether or not their stimulation by cAMP/IGF1 requires GATA4/6 or whether GATA factors only enhance the effects of FSH in gene expression. Thus, cAMP/IGF1 stimulated the expression of Cyp19a1, inhibin-α, Papp-a, and Prkar2b in WT cells, but not in GATA4/6 knockout cells (Fig. 22A). In contrast, the increase of Cyp11a1 expression induced by cAMP/IGF1 was not affected by the absence of GATA4/6 expression. Finally, cAMP/IGF1 stimulated the expression of Lhcgr and Vcan in both WT and GATA4/6 knockout cells; however, full stimulation of these genes was only attained in WT cells. The results also suggest that the basal expression of several genes is affected by GATA4 and GATA6. In untreated cells, the knockdown of these factors decreased the basal expression of Cyp19a1, Vcan, inhibin-α, PlxnC1, and Grem2 and increased the basal expression of IGFBP4 and Papp-a. Thus, the upregulation of IGFBP4 in the absence of GATA factors observed *in vivo* and *in vitro* suggests that GATA4 and GATA6 are required to maintain low expression levels of this binding protein.

Noteworthy, we observed that the expression of Comp, PlxnC1, and Grem2 was stimulated by eCG *in vivo* but inhibited by dbcAMP in the *in vitro* experiments. These results suggest that FSH may not be the main factor regulating the expression of Comp, PlxnC1 and Grem2. In fact, the expression of Grem1 and Grem2 is stimulated by growth differentiation factor 9 (GDF9) (115). Our findings suggest that GATA4 and GATA6 may mediate this effect of GDF9.

These findings suggest that knockdown of GATA4 and GATA6 directly and indirectly affect gene expression in GCs (Fig. 22B). Direct effects occur when basal or FSH-induced stimulation of gene expression requires GATA factors whereas indirect effects are mediated by the reduction of FSHR expression in the absence of GATA factors. Thus far, only Cyp11a1 meets the latter criterion.

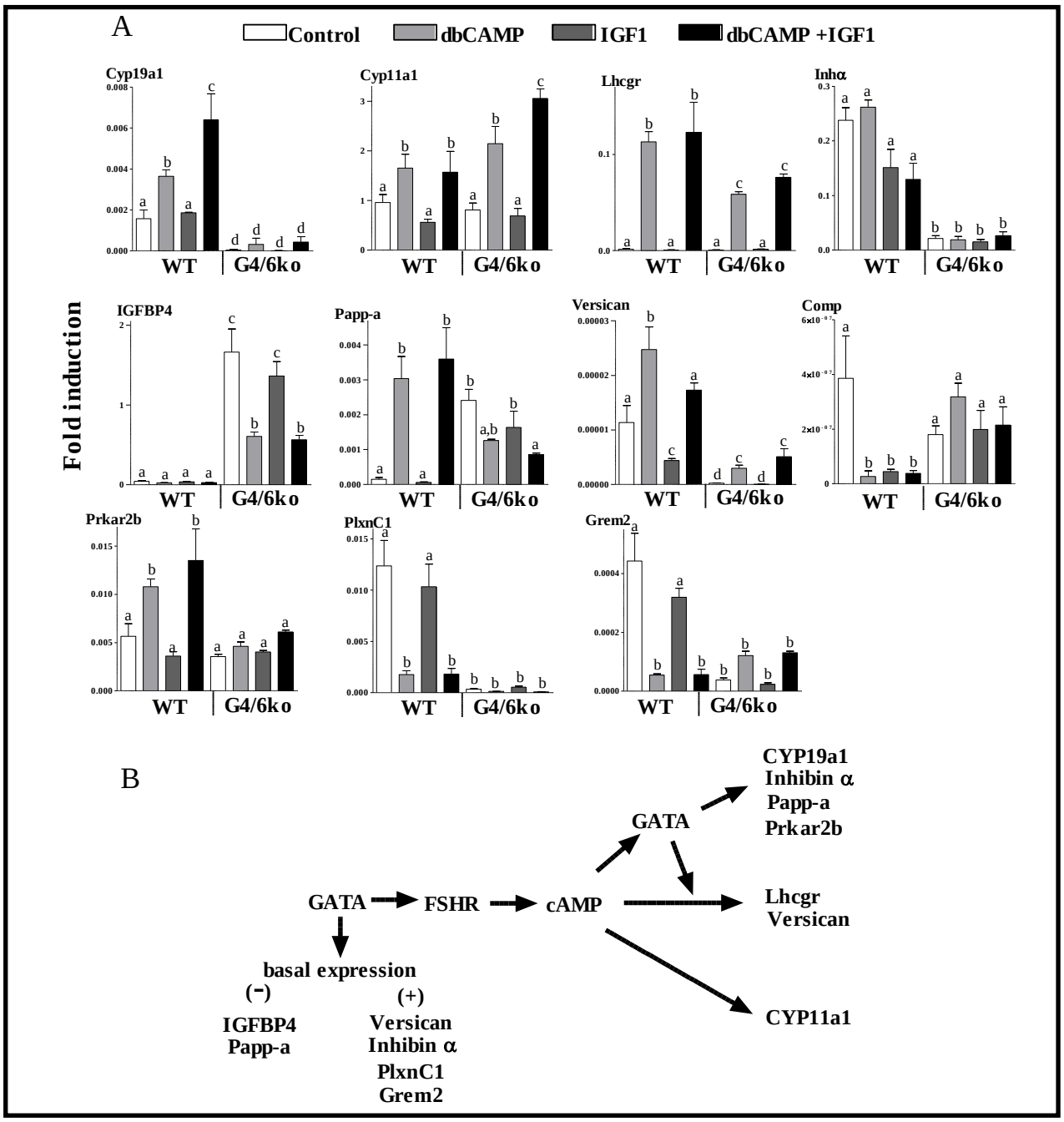


Figure 22. Role of FSH Receptor Silencing on the Regulation of Gene Expression by GATA Factors.

A) Effect of dibutyryl cAMP (a cAMP analog) and/or IGF1 on the expression of selected genes in WT or GATA4 and GATA6 deficient granulosa cells obtained from immature WT or GATA4F/F;GATA6F/F animals. Granulosa cells were cultured for 24 hs with a Cre-recombinase expression adenovirus at a multiplicity of infection of 10. Cells were then treated for 48 hs with vehicle, dbcAMP (1mM), IGF1 (50 ng/mL) or their combination. *Columns* represent the mean ± SEM of six different samples. Columns with different letters differ significantly (one-way ANOVA, Tukey test). **B**) General scheme indicating genes regulated directly by GATA factors or indirectly via the inhibition of FSHR expression.

D. Conclusions

In chapter III, we demonstrated differential, but also overlapping actions of GATA4 and GATA6 in the ovary. Thus, it was shown that $G6^{gcko}$ mice are fertile, that $G4^{gcko}$ mice are subfertile, and that the absence of both factors causes infertility. In this chapter, we intended to determine why GATA4 is more dominant than GATA6 and to examine the mechanisms that cause infertility in the absence of both factors. In answering these questions, this report revealed that more genes were regulated by GATA4 than by GATA6 and that even more genes were affected when both factors were absent. These findings also provide a possible answer to our initial question regarding the compensatory role that GATA4 and GATA6 have in the ovary and suggest that genes involved in the final stages of follicle maturation might be controlled by both GATA4 and GATA6. This observation is supported by the fact that many genes are only affected by the absence of both factors. This may represent an evolutionary adaptation to guarantee the normal development of preovulatory follicles. In addition, our findings demonstrated that the expression of the FSHR decreases only when GATA4 expression was targeted ($G4^{gcko}$ or $G4/6^{gcko}$), but not in the absence of GATA6 alone. Because the FSHR is essential for the differentiation of GCs, this finding may explain the predominant role GATA4 in ovarian folliculogenesis.

Deletion of GATA4 and GATA6 in GCs demonstrated not only the crucial role that these factors have in ovarian function and female fertility, but also offer a unique experimental paradigm to examine genes and pathways involved in the regulation of antral follicle formation. In this regard, this report indicates that the main and probably one of the early defects occurring in GATA deficient follicles is the inhibition of proliferation and differentiation programs needed for the formation of large pre-ovulatory follicles. A direct consequence of these two actions is the lack of follicular antrum formation. A key step in antrum formation is the production of hyaluronan and versican both of which generate an osmotic gradient that draws fluid from the thecal vasculature (151). In the absence of GATA factors, no changes in the expression of hyaluronan synthases were observed; however, the expression of versican was abolished. Since versican is a large proteoglycan that crosslinks with hyaluronan (152,153), it may

contribute greatly to the osmotic potential of the follicular fluid and to the formation of the antrum. Therefore, lack of versican expression may explain, at least in part, the lack of antral follicle formation observed in GATA conditional knockout animals.

Limited information is available regarding the transcriptional defects that lead to the halt in folliculogenesis observed in animals lacking FSHβ, FSHR, or IGF1 (12,13,38). The lack of GATA4 and GATA6 in GCs decreases FSHR expression and increase genes known to reduce IGF1 receptor signaling. Therefore, the genome-wide changes in gene expression observed in GATA4/6^{gcko} animals could shed some light on the mechanisms involved in the deregulation of the folliculogenesis process in the absence of FSH and/or IGF1 signaling. It should also be mentioned that although GATA4 and GATA6 are transcription factors, indirect and non-transcriptional effects might account for the phenotypes observed in the absence of these factors in GCs.

In conclusion, our results suggest that GATA4 regulates, directly or indirectly, a greater number of genes than GATA6. However, since an even greater number of genes are affected by the absence of both factors, we propose that these factors functionally compensate for each other during GC differentiation.

V. CONDITIONAL DELETION OF GATA4 AND GATA6 AT OVULATION IN THE OVARY IMPAIRS PROGESTERONE SYNTHESIS AND LEADS TO FEMALE INFERTILITY

A. Introduction

Thus far, we have shown that deletion of the transcription factors GATA4 and GATA6 in the granulosa cells of early antral follicles results in anovulation and infertility (Chapter III). These defects are a consequence of a block in folliculogenesis that prevents the formation of preovulatory follicles (83). Lack of follicle development precludes studies to examine the role of GATA4 and GATA6 in luteal cells where GATA factors are also expressed. Therefore, it is not known if these factors are involved in the regulation of luteal function *in vivo*.

Steroidogenesis is essential for fertility as it produces the estrogen and progesterone needed to maintain uterine function and pregnancy. The findings in the previous chapter demonstrated that GATA factors are able to regulate a number of genes in the steroidogenesis pathway including Cyp1b1, Cyp19a1 and Cyp11a1. In particular, Cyp11a1 and StAR are necessary for progesterone synthesis and are known targets of GATA factors. StAR promoter activity is upregulated by GATA4 in luteinized porcine granulosa cells and we have demonstrated that Cyp11a1 expression in preovulatory granulosa cells requires GATA factors ((72), Chapter III and Chapter IV). As progesterone synthesis from the corpus luteum (CL) is necessary for implantation and maintenance of pregnancy (8,10), the role that GATA factors have in the regulation of luteal function needs to be assessed.

The aim of this chapter was to determine the effect of the knockdown of GATA4 and GATA6 at ovulation prior to CL formation. To delete GATA4/6 in the CL, mice expressing Cre recombinase driven by the progesterone receptor (PR) promoter, which is upregulated in granulosa cells at ovulation, were crossed with mice containing single or combined floxed alleles for GATA4 and GATA6. We found that GATA4/6-PR-Cre ($G4/6^{prko}$) animals are infertile. In addition, we show for the first time that GATA factors are necessary for progesterone production in the CL. This finding is supported by a decrease in plasma progesterone and a decrease in the expression of steroidogenic enzymes needed for

progesterone production. In addition, we demonstrate that implantation could be rescued by treating G4/6^{prko} animals with progesterone. Although PR-Cre may drive the silencing of GATA4/6 in other tissues including the oviduct and the uterus, our findings suggest that these transcription factors are required in the ovary for progesterone synthesis in the CL of mice.

B. Results

1. Disruption of *GATA4* and *GATA6* Genes in Progesterone Responsive Tissue

The Cre-Lox system was used to knockdown GATA4 and GATA6 expression in progesterone-responsive tissues as no corpora lutea specific Cre-recombinase animal exists. To selectively disrupt GATA expression in progesterone-responsive cells, GATA4F/- and/or GATA6F/- mice were crossed with transgenic mice expressing Cre driven by the progesterone receptor (PR) promoter. In PR-Cre animals, Cre is expressed in the pituitary, mammary, uterus, oviduct and corpora lutea (82). In this study, we used GATA4F/-/GATA6F/F; PR-Cre (G4/6^{prko}) animals in all experiments unless a specific genotype is indicated. Wildtype animals were used as controls. The knockdown of GATA4 and GATA6 at the level of mRNA was confirmed using whole ovaries, oviducts and uteri from superstimulated animals that had received 7.5 IU eCG for 48 hs followed by 7.5 IU hCG for 96 hs (Fig. 23). GATA4 and GATA6 mRNA levels were significantly reduced in the ovaries and oviducts obtained from G4/6^{prko} animals. However, low basal mRNA levels of GATA4 and GATA6 expression were found in the uteri of control animals and G4/6^{prko} animals had undetectable changes in GATA6 expression, while GATA4 expression in the uterus was found to be significantly increased.

In order to confirm the knockdown of GATA factors in G4/6^{prko} animals, immunohistochemical analysis was done (Figure 24). Immunostaining for GATA4 (Fig. 24A, 24E) and GATA6 (Fig. 24C, 24G) in control and G4/6^{prko} (Fig. 24B, 24F; GATA4 and 24D, 24H; GATA6) ovaries indicated a decrease of GATA expression in the CL of G4/6^{prko} but not in granulosa cells of developing follicles. Additionally, the luminal epithelium of the oviduct and uterus strongly stained for GATA4 (Fig. 24A,

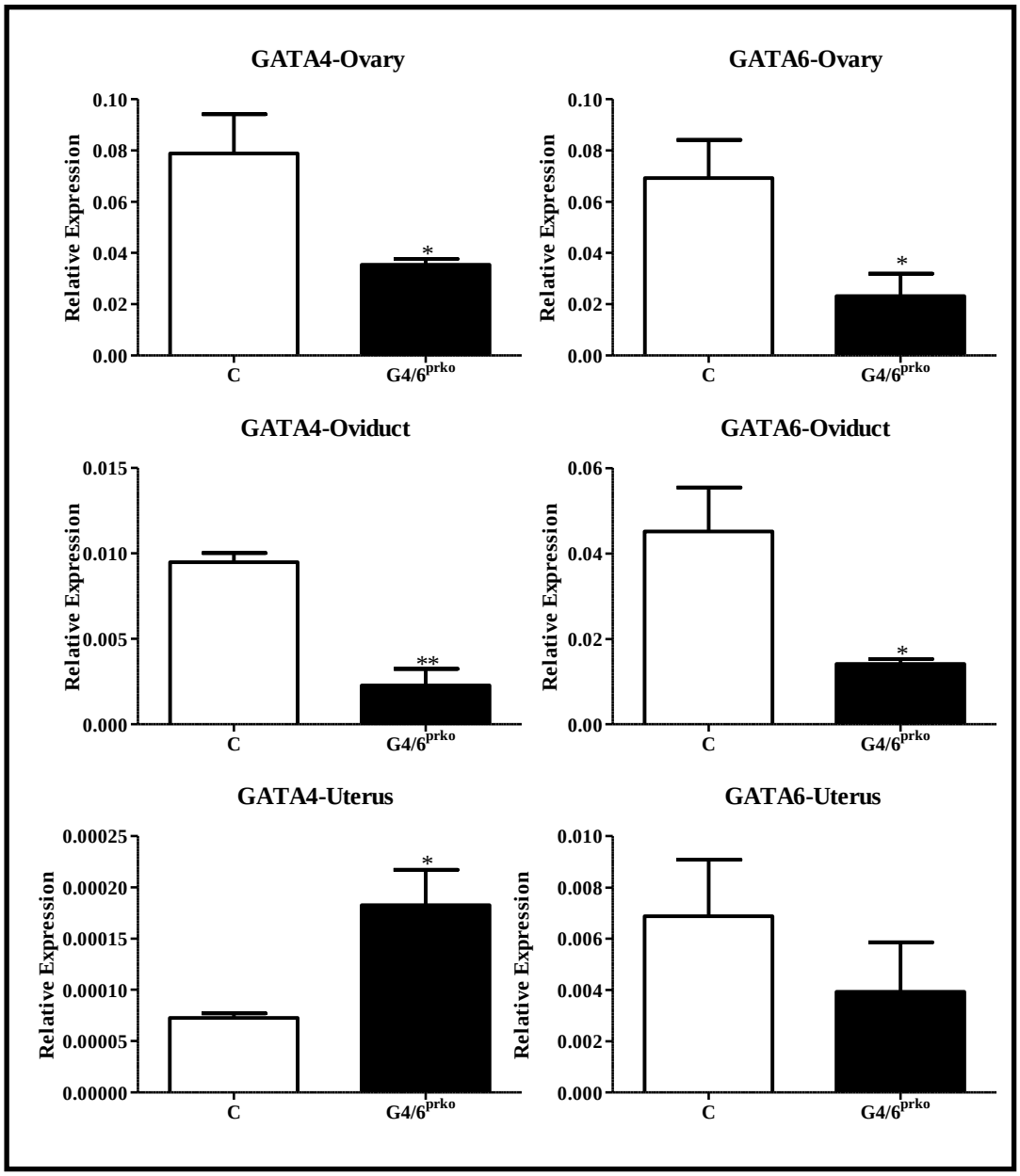


Figure 23. In Vivo Knockdown of GATA mRNA Expression in Ovaries and Oviducts of GATA4/6^{prko} Animals

mRNA expression of GATA4 (*top*) and GATA6 (*bottom)* from eCG-treated (48 hs), hCG-treated (96 hs) animals. mRNA was isolated from whole ovaries, oviducts and uteri. mRNA expression is relative to mouse ribosomal L19 (the average of four or more samples per genotype is shown). *, $P<0.05$, **, $P < 0.01$

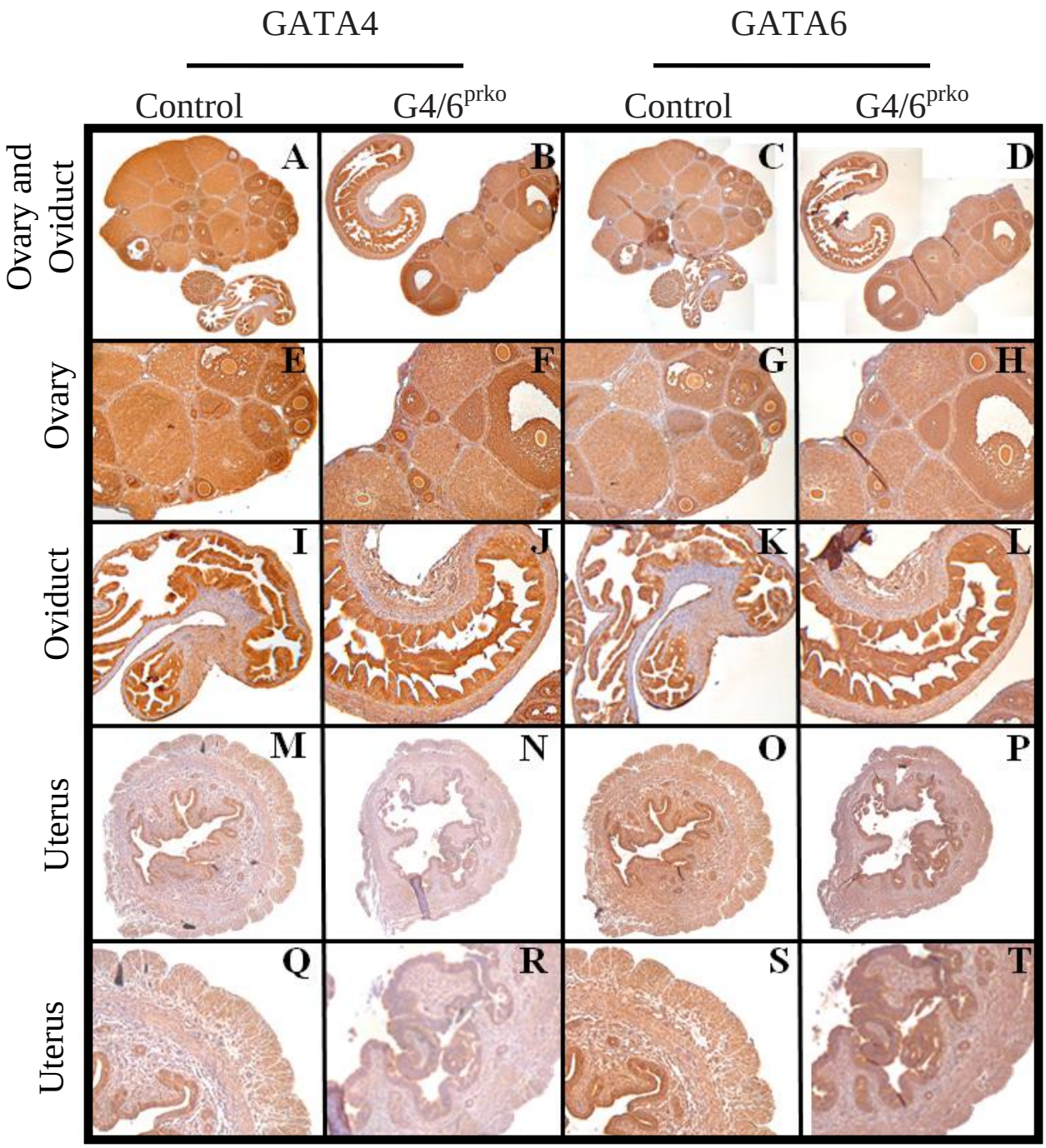


Figure 24. Knockdown of GATA Factor Proteins in GATA4/6^{prko}

Immunohistochemistry of control and GATA4/6^{prko} animals treated with eCG for 48 hs followed by hCG for 96 hs. Control ovary (**A, E**), oviduct (**A, I**), and uterus (**M, Q**) stained for GATA4. GATA4/6^{prko} ovary (**B, F**), oviduct (**B, J**), uterus (**N, R**) stained for GATA4. Control ovary (C, G), oviduct (**C, K**) and uterus (**O, S**) stained for GATA6. GATA4/6^{prko} ovary (**D, H**), oviduct (**D, L**) and uterus (**P,T**) stained for GATA6. Smaller pictures at a magnification of 20x, enlarged pictures are 40x. One representative picture is shown for each tissue and genotype. N=3.

24I; oviduct, 24M, 24Q; uterus) and GATA6 (Fig. 24C, 24K; oviduct, 24O, 24S; uterus) while the stroma of the uterus expressed less GATA protein in control animals. The expression of both factors appeared slightly reduced in the G4/6^{prko} oviduct (Fig. 24B, 24J; GATA4, 24D, 24L; GATA6). Expression of GATA4 (Fig. 24N, 24R) and GATA6 (Fig. 24P, 24T) appeared highly reduced in the luminal epithelium and stroma of uterus of the G4/6^{prko} mice. Thus, the use of PR-Cre seems to lead to a decrease in GATA factor expression in the ovary; however, we found conflicting results in the knockdown of these factors in the oviduct and uterus.

2. GATA4 and GATA6 at Ovulation are Essential for Female Fertility

The fertility of mice lacking GATA4 and/or GATA6 in progesterone-responsive tissues was tested by mating control or experimental females with males of proven fertility for 6 months. Both G4^{prko} and G6^{prko} animals had a significant decrease in the number of pups per litter when compared with controls. In marked contrast, five out of six double-knockout females were infertile (Fig. 25A). One G4/6^{prko} animal had one litter but no subsequent pregnancies. These results demonstrate that the expression of both GATA4 and GATA6 in progesterone-responsive tissues is essential for female fertility.

Next, the length of the various phases of the estrous cycle was compared between control, G4^{prko}, G6^{prko}, and G4/6^{prko} animals (Fig. 25B). The results showed that there were no significant differences in the cycling of the knockouts compared to control. This suggests that the loss of GATA factors most likely has no effects on ovarian cyclicity or gonadotropin levels. To determine if ovulation was affected, the response of control and G4/6^{prko} animals to a superovulation protocol was examined. Control and G4/6^{prko} females were stimulated with eCG for 48 hs followed by hCG administration. The presence of oocytes in the oviducts was determined 17 hs after hCG treatment. GATA4/6^{prko} animals had comparable number of oocytes released after superovulation to control animals (Fig. 26A). This suggests that the infertility phenotype of the G4/6^{prko} is not due to a lack of cyclicity or ovulation.

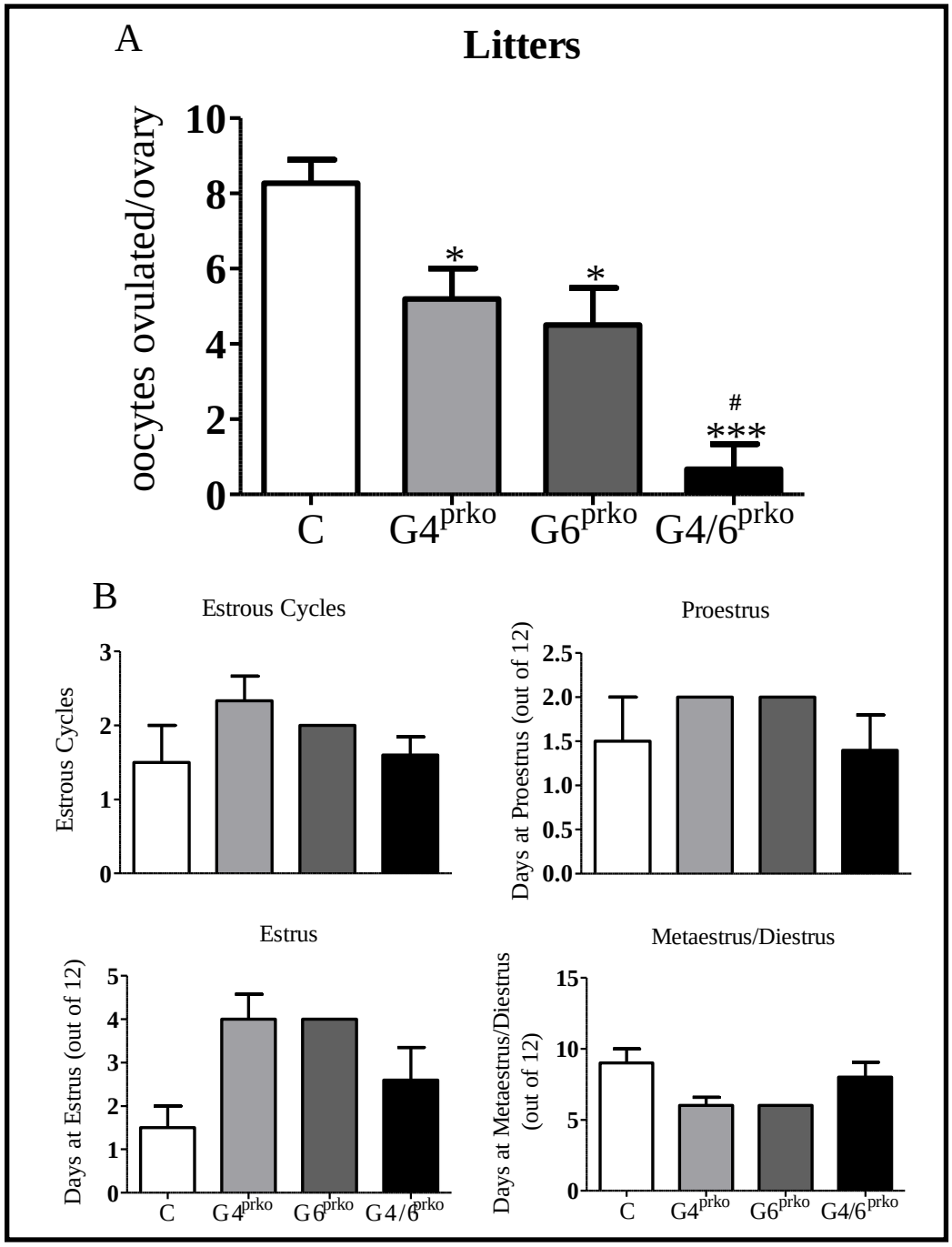


Figure 25. Loss of GATA Factors Decreases Fertility but Does Not Impair the Estrous Cycle in G4/6^{prko} mice

A) Adult (~d42) control, GATA4^{prko}, GATA6^{prko} and GATA4/6^{prko} females were paired with fertile males for 6 months and the number of pups per litter from those pairings was assessed. At least 4 animals used for each genotype. The *columns* represent the average number of pups per litter ± SEM. #, One of 6 animals had one litter but no subsequent litters. *, $P < 0.05$, ***, $P<0.001$. **B**) The estrous cycle was tracked over the course of 12 d in control, G4^{prko}, G6^{prko}, and G4/6^{prko} adult females. Each estrous cycle was broken into three stages of proestrus, estrus, and metestrus/diestrus. The *columns* represent the average number of days spent in each stage of the estrous cycle ± SEM.

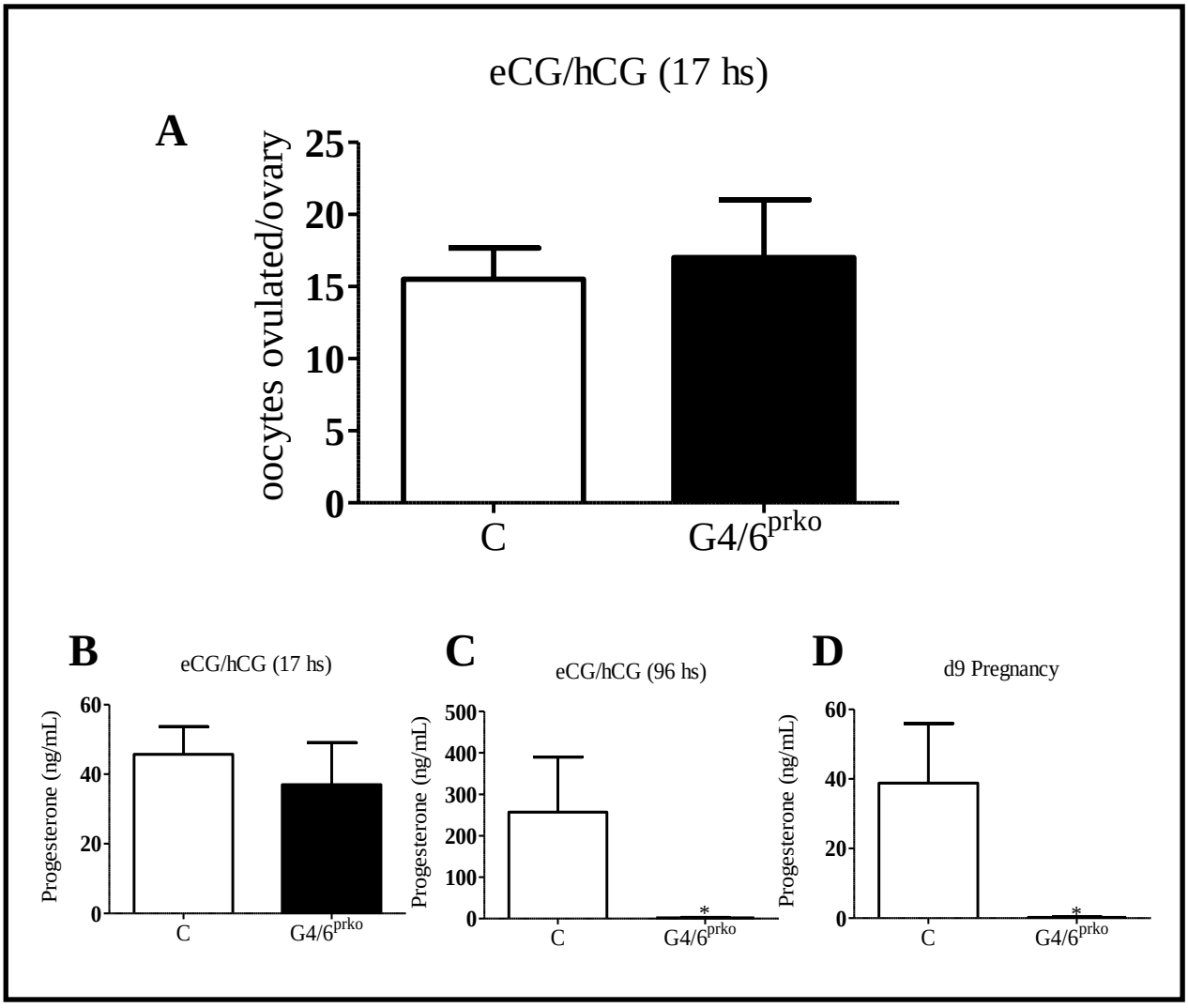


Figure 26. GATA Knockdown Does Not Affect Ovulation but Reduces Plasma Progesterone Levels

A) Oocytes were obtained and counted from the oviducts of control (C) and GATA4/6^{prko} (G4/6^{prko}) mice superovulated with eCG (48 hs) followed by hCG (17 hs). N≥3. Plasma progesterone levels determined in superovulated animals (**B**), animals treated with eCG (48 hs) and then hCG (96 hs) (**C**) or animals at day 9 of pregnancy (**D**). *Columns* represent the average ± SEM. N≥3. *, $P \leq 0.05$.

As G4/6^{prko} animals cycle and ovulate normally, we examined if the infertile phenotype was a result of an impairment in the maintenance of pregnancy. Consequently, the circulating levels of progesterone were examined in control and double knockout animals. When compared with control animals, no significant changes in progesterone levels in the plasma of G4/6^{prko} animals were observed 17 hs after hCG administration (Fig. 26B). However, plasma progesterone levels were significantly lower in G4/6^{prko} 96 hs after hCG treatment when compared with controls (Fig. 26C). Similarly, plasma progesterone levels were significantly lower in the G4/6^{prko} animals 9 days after a vaginal plug was found when compared to control animals on day 9 of pregnancy (Fig. 26D). This suggests that the infertility phenotype of the G4/6^{prko} females might be due to decreased progesterone levels, which are inadequate to sustain implantation and pregnancy.

As progesterone levels are low in the GATA4/6^{prko} animals, the mRNA levels of the steroidogenic enzymes important for progesterone synthesis as well as other genes important for CL function were determined. We found that mRNA levels of Cyp11a1 and StAR were both significantly decreased in the G4/6^{prko} (Fig. 27). Prolactin receptor (Prlr) and Lhcgr also had low expression in the G4/6^{prko} animals, although these data were not significantly different from that of control. Lastly, we found that the luteal expression of Vcan and the transcription factor Pax2 in the G4/6^{prko} was significantly decreased. These findings suggest that the knockdown of GATA factors results in a decrease in the expression of enzymes involved in the synthesis of progesterone as well as impacts the extracellular matrix and development pathways.

In order to confirm the effect of GATA deletion on the expression of enzymes involved in progesterone synthesis found *in vivo* as well as genes important for CL function, we next cultured luteal cells from control and G4F/F; G6F/F animals treated with eCG 48 hs followed by hCG 7 hs as hCG will induce the luteinization process as well as ovulation. After plating, luteinized granulosa cells were transfected with AdCre at a MOI:10 to knock down GATA factors *in vitro* and cultured for 72 hs. The knockdown of GATA factors was confirmed in GATAF/F; GATA6F/F cells treated with AdCre when compared with control cells treated with AdCre (Fig. 28). Similar to the *in vivo* findings, Prlr, StAR and

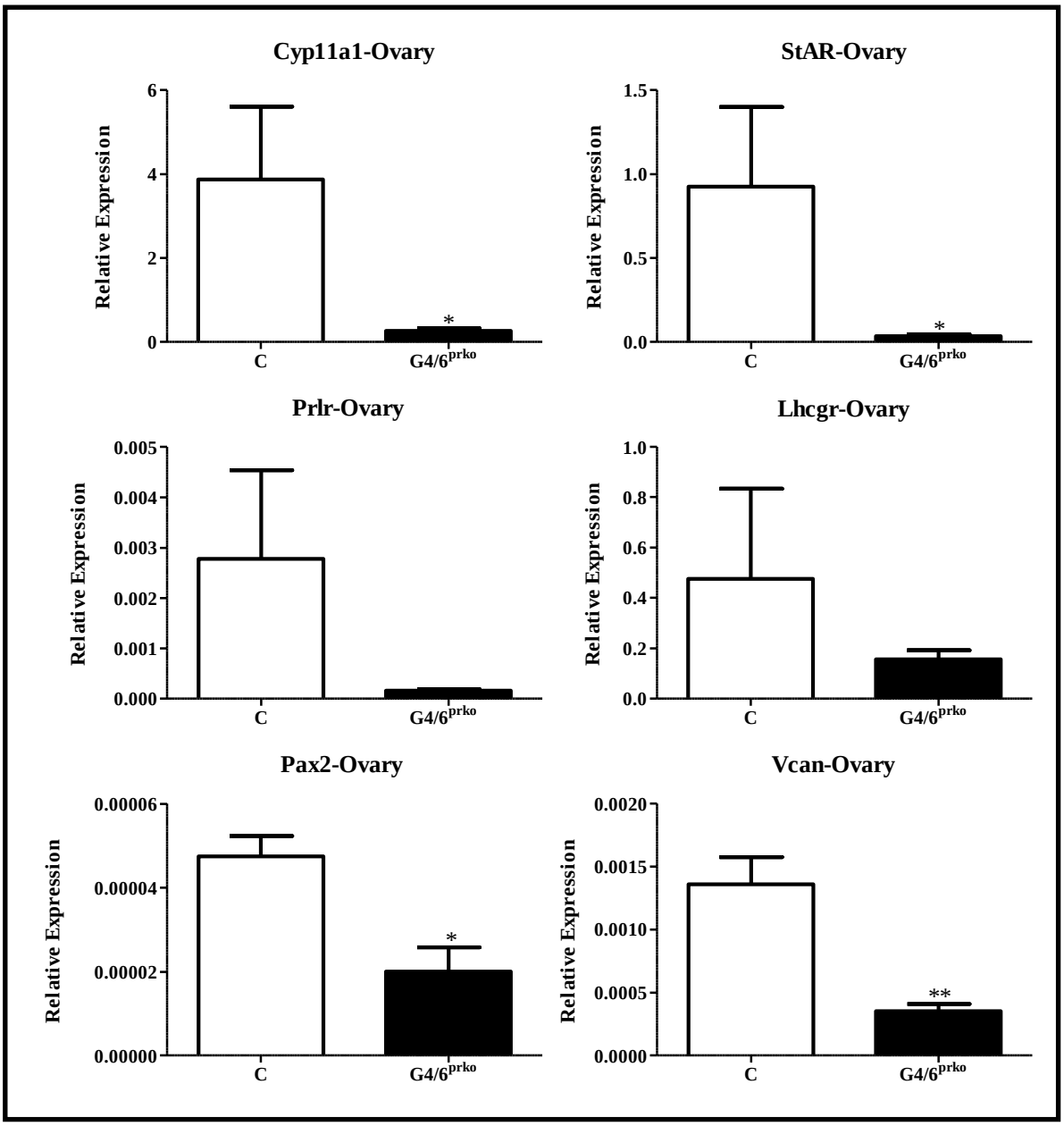


Figure 27. GATA Knockdown Regulates mRNA Expression of Ovarian Genes *In Vivo*

Whole ovaries from control and GATA4/6^{prko} animals treated with eCG (48 hs) followed by hCG (96 hs). mRNA expression is relative to mouse ribosomal L19. *Columns* represent the average ± SEM. N=4 for all columns. *, $P<0.05$, **, $P< 0.01$.

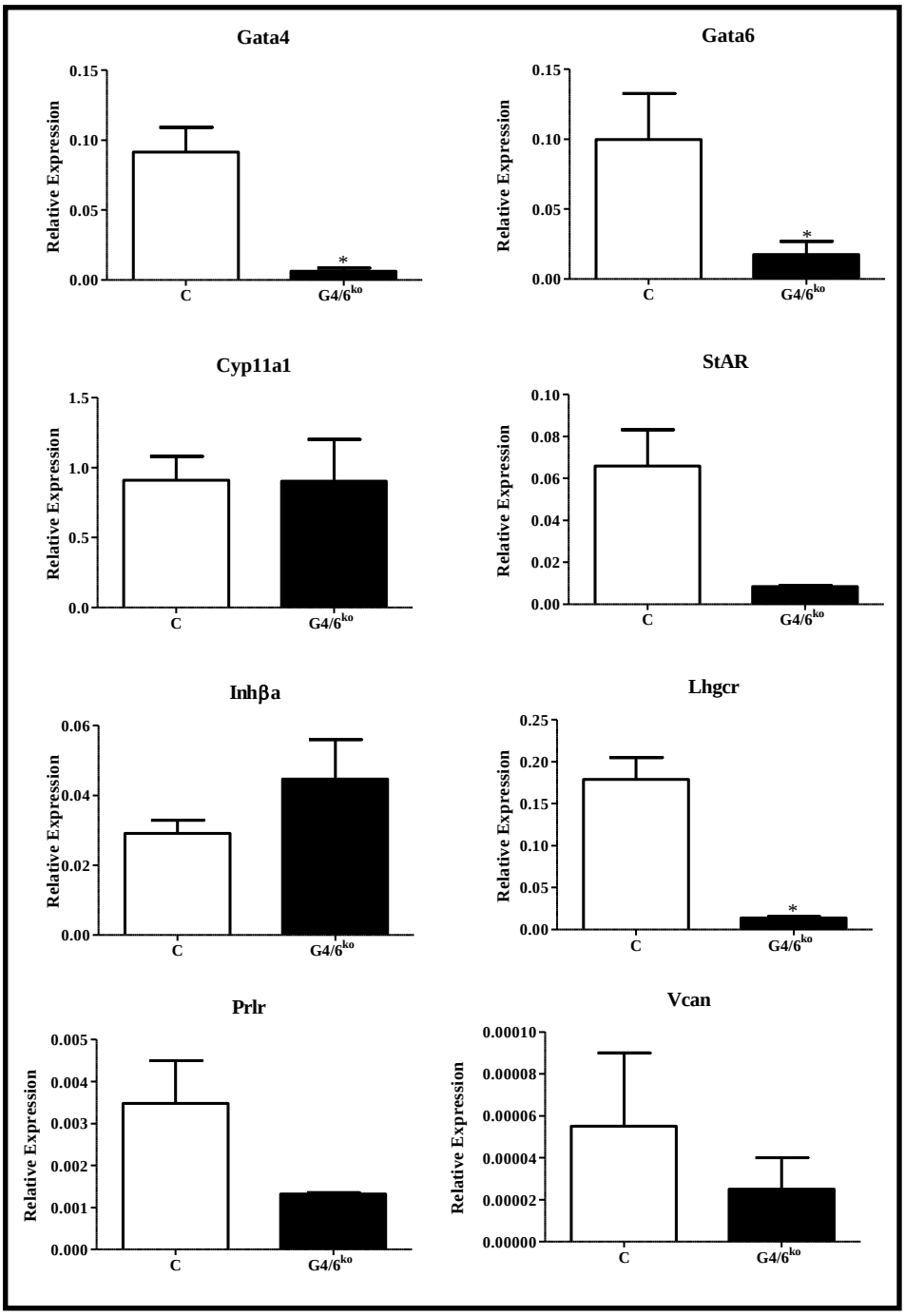


Figure 28. GATA Knockdown Regulates mRNA Expression of Ovarian Genes *In Vitro*

Luteal cells were isolated from control (C) and GATA4F/F; GATA6F/F ($G4/6^{ko}$) animals treated with eCG (48 hs) followed by hCG (7 hs). All cells were treated with adCre (MOI:10) and cultured for 72 hs. *Columns* represent the average ± SEM. Samples were in duplicate or triplicate. N=2. *, $P<0.05$

Vcan tended to decrease with the loss of GATA factors. Lhcgr was significantly decreased in the G4/6^{ko} cells which also supports the *in vivo* findings. However, Cyp11a1 did not decrease *in vitro*. Additionally, Inhβa tended to increase *in vitro*. These findings support the *in vivo* results in which key genes necessary for luteal function tend to decrease with the loss of GATA4 and GATA6.

3. Loss of GATA Factors Affects the Function of the Corpora Lutea but Not the Structure

The corpus luteum is the only source of progesterone in pregnant mice, so the structural characteristics of this gland were examined in G4/6^{prko} animals using hematoxylin and eosin staining. The number and structure of the CL in G4/6^{prko} animals after superstimulation of eCG/hCG 96 hs (Fig. 29B) or at day 9 of pregnancy (Fig. 29D) was comparable with controls (Fig. 29A and 29C respectively). One minor abnormality was that there was a higher incidence of trapped oocytes within the CL of the knockout animals than control. Because structure doesn't always denote problems with function, we also stained ovaries of control and G4/6^{prko} treated with eCG/hCG 96 hs in Figure 30 for PCNA (a proliferation marker), cleaved caspase 3 (an apoptosis marker) and VEGF (a vasculature marker). There was no alterations in the staining for any of these proteins suggesting that alterations in progesterone synthesis was not a result of corpora lutea undergoing luteolysis at a higher rate, having impaired cell proliferation or lacking the vasculature to maintain corpus luteum function.

Because G4/6^{prko} animals had reduced progesterone levels but ovulate normally, we next examined whether implantation was affected in these animals. In G4/6^{prko} animals, no sites of implantation could be seen at day 9 of pregnancy (Fig. 31A, left). Consequently, we examined whether the lack of implantation could be rescue by progesterone administration. For this purpose, G4/6^{prko} females were mated with males of proven fertility and the presence of vaginal plugs was examined daily. Vaginal plugs were found in all animals, suggesting that normal mating had occurred in the control and G4/6^{prko} females. The day the plug was detected was considered day 1 of pregnancy. Upon detection of a plug, G4/6^{prko} animals were injected with 3mg/ml progesterone sc daily from day 1 until

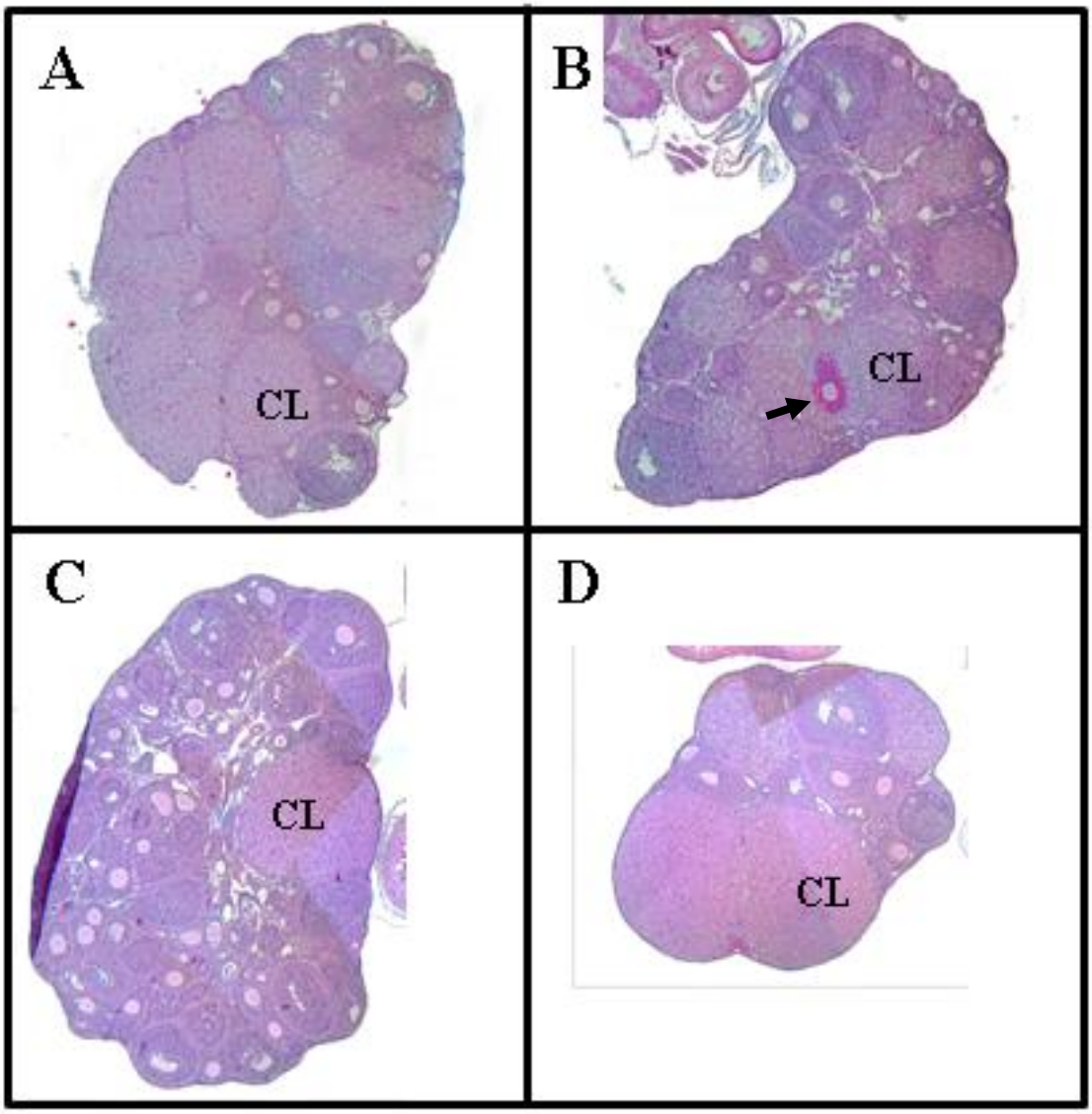


Figure 29. Corpora Lutea are Present in GATA4/6^{prko}

Hematoxylin and eosin staining of representative ovaries. Control (**A**) and G4/6^{prko} (**B**) ovaries from animals treated with eCG/hCG (96 hs). Control (**C**) and G4/6^{prko} (**D**) ovaries from animals at day 9 of pregnancy. CL: corpus luteum, arrow: oocyte trapped in CL. N=3

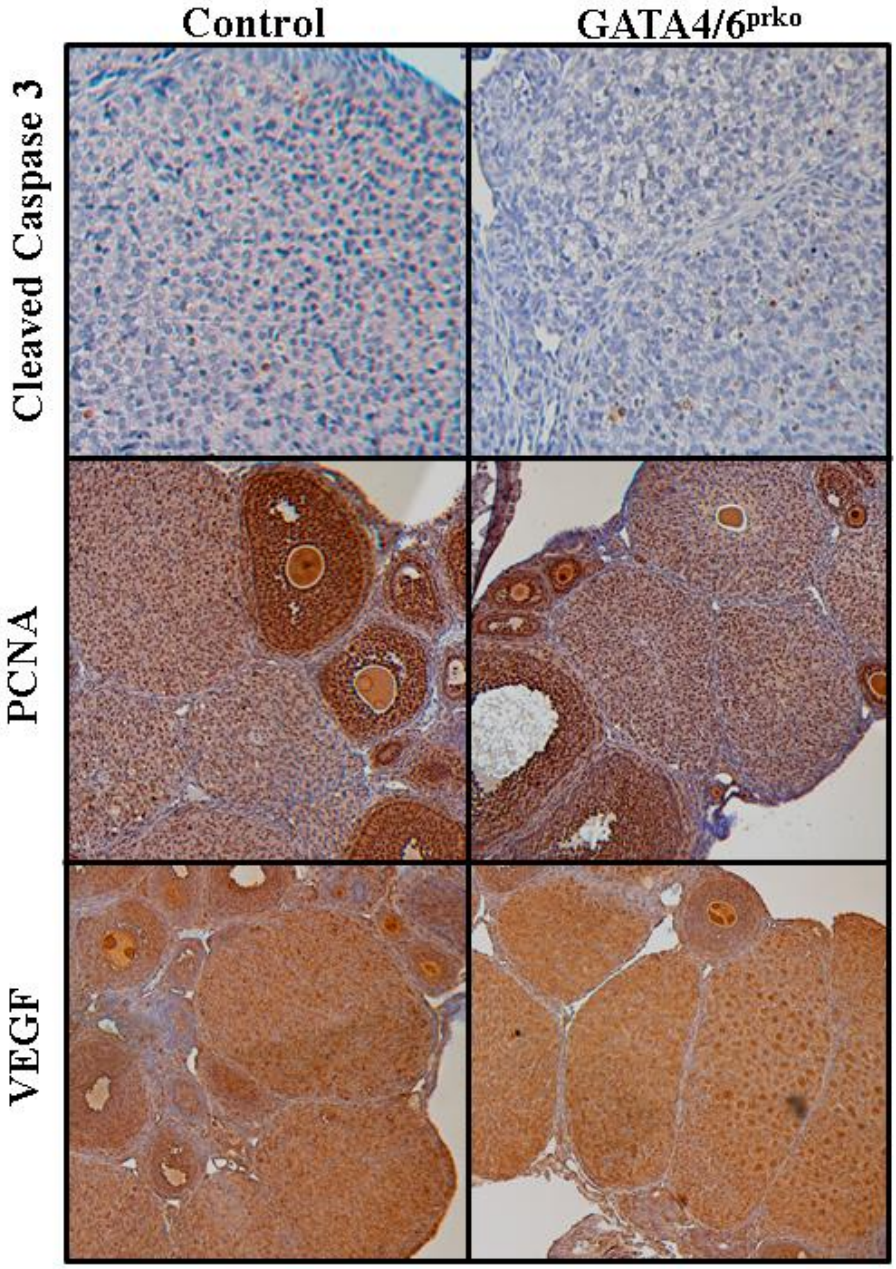


Figure 30. Knockout of GATA Factors Does Not Affect Proliferation, Apoptosis or Vascularization in the G4/6^{prko} Ovary

Immunohistochemistry of control and GATA4/6^{prko} ovaries from animals treated with eCG/hCG (96 hs). Ovaries were stained (brown) for cleaved caspase 3 (apoptosis marker), PCNA (proliferation marker) and VEGF (vascularization marker) as denoted by the label on the left. Representative ovaries are shown, N=3.

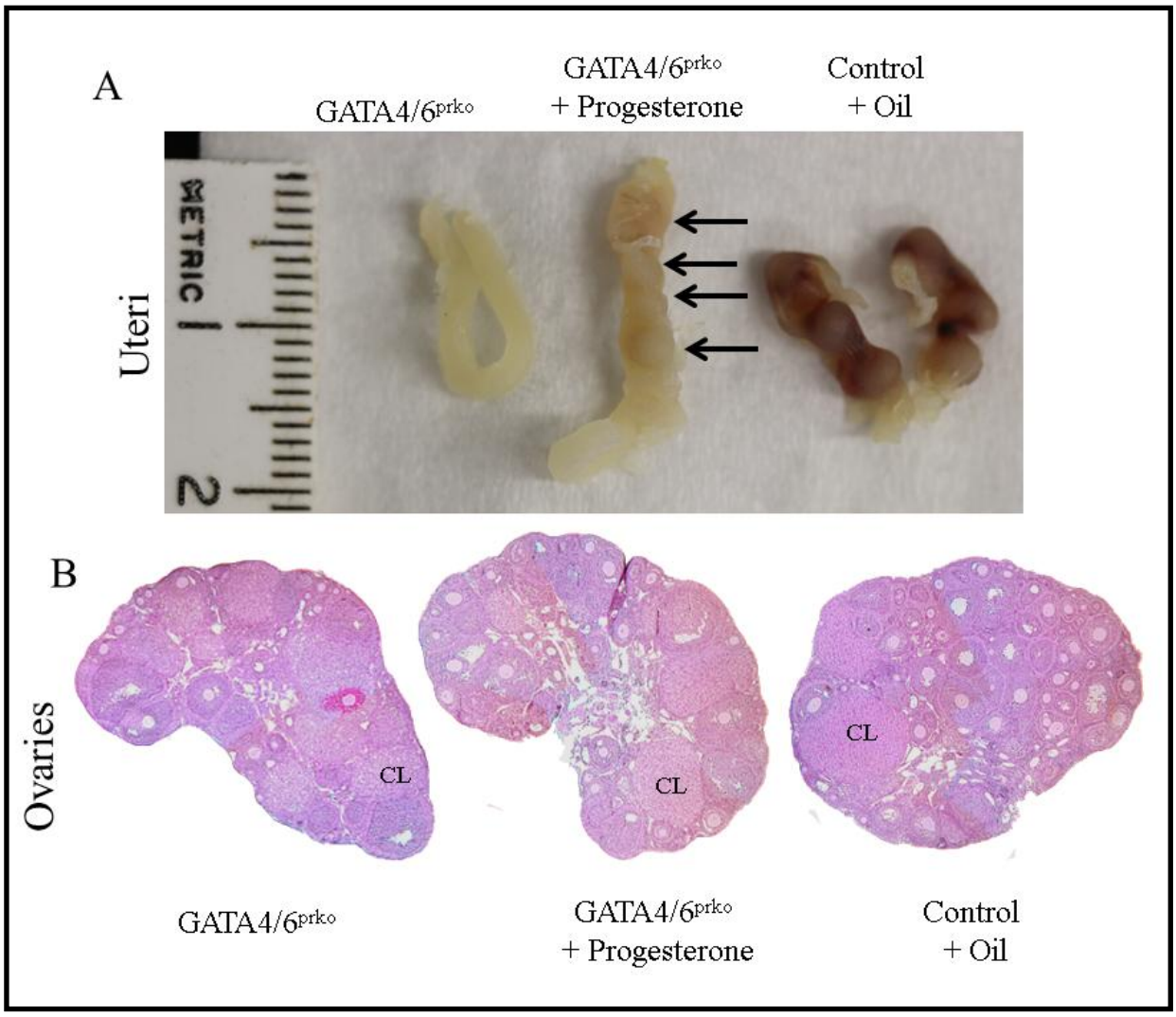


Figure 31. Progesterone Treatment can Rescue Implantation in the GATA4/6^{prko}

Adult females were paired with proven males. Presence of a plug at D1 of pregnancy was followed by 3mg/ml daily injections of progesterone (P4) or equivalent amount of sesame oil (Oil) until sacrificed at day 9 of pregnancy. **A**) Uteri fixed in formalin. **B**) H&E of ovaries. Representative tissues are shown. N=2.

day 9 of pregnancy, control animals were injected with an equivalent amount of sesame oil. As expected, implantation was normal in control animals (Fig. 31A, 31B, right). Administration of progesterone rescued implantation in 1 out of 2 G4/6^{prko} animals tested (Fig. 31A, middle). However, the implantation sites found in this G4/6^{prko} mouse appears unhealthy compared to control (Fig. 31A, right) as the fetuses were smaller and there appears to be less vascularization. Although, these are not conclusive results, they suggest that implantation could be rescued by exogenous progesterone, indicating that impairment of luteal function may account, at least in part, for the infertility phenotype of the G4/6^{prko}.

4. Lack of GATA Factors Alters Oviductal Morphology

The knockout of GATA factors in the ovary appears to significantly contribute to the G4/6^{prko} infertility phenotype; however, there were other tissues impacted with the knockdown of GATA in using the PR-Cre mice. Hematoxylin and eosin staining of the oviducts of G4/6^{prko} mice either after eCG/hCG (96 hs) treatment (Fig. 31B) or from day 9 of pregnancy (Fig. 32D) revealed structural abnormalities compared to similar controls (Fig. 32A and 32C). In particular, G4/6^{prko} oviducts of have cells within the tubules, suggesting tubal occlusion. Additionally, there is an increased size of the columnar epithelium and loss of polarity in the epithelial cells in the knockouts. Thus, the nuclei are no longer localized centrally but are rather closer to the base of the cells. These structural abnormalities could influence the progression of the oocyte to the uterus and impair fertility.

To determine if function of the oviduct had been altered as well as the morphology, mRNA expression in oviducts from control and G4/6^{prko} animals treated with eCG/hCG 96 hs was assessed (Fig. 33). As GATA factors are mainly expressed within the epithelium of the oviduct, the expression of oviduct-specific glycoprotein 1 (OVGP1) and paired box 2 (Pax2), two genes specifically expressed in the epithelium of the oviduct (154,155) was assessed. OVGP1 has an important role in the oocyte-sperm interaction as it modifies the zona pellucida surrounding the oocyte to help prevent polyspermy (156). Pax2 is a gene highly expressed in the secretory cells of the oviduct and has been shown to be

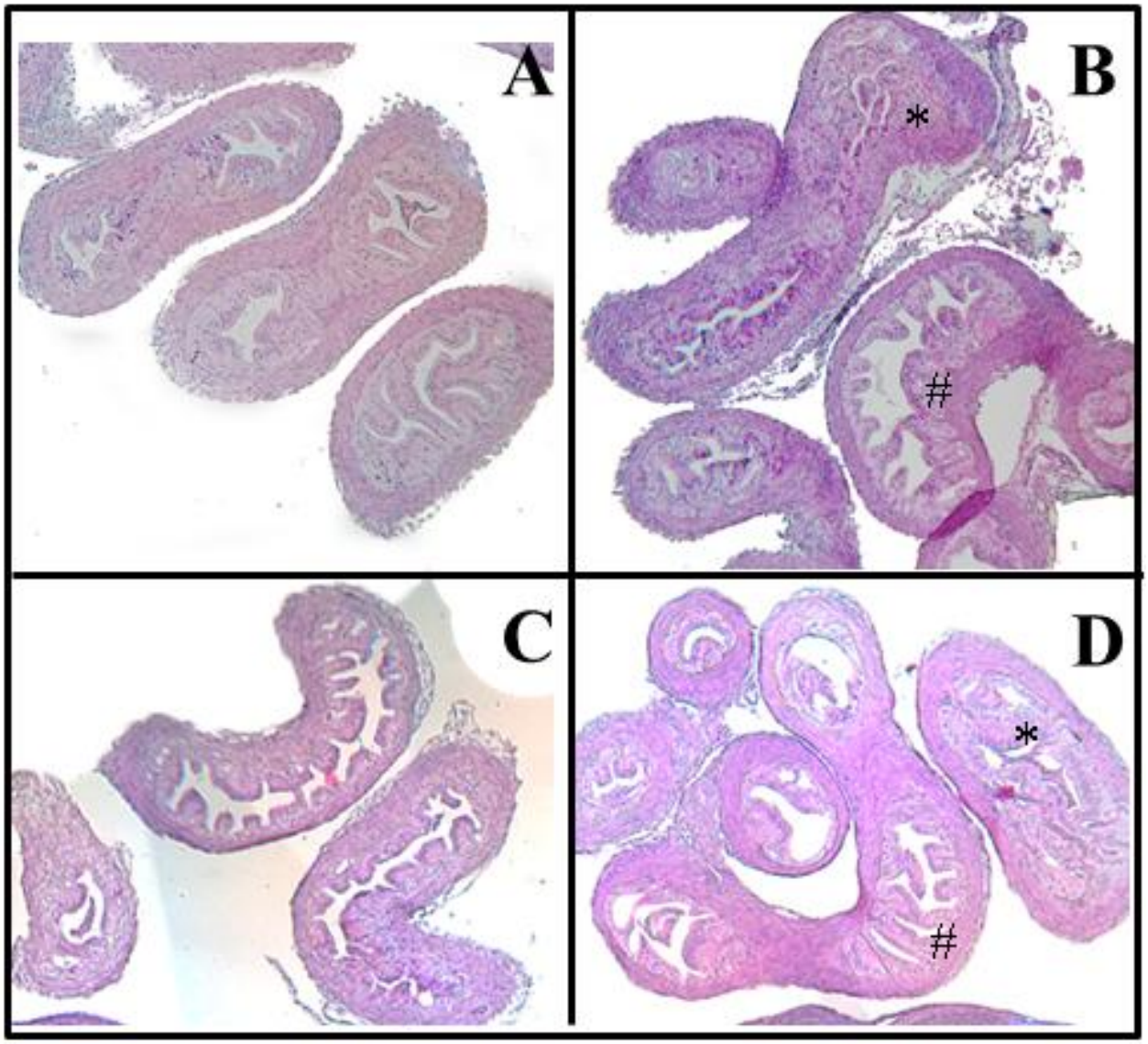


Figure 32. Loss of GATA Factors Leads to Abnormal Oviductal Morphology

Hematoxylin and eosin staining of representative oviducts. Control (**A**) and G4/6^{prko} (**B**) ovaries from animals treated with eCG/hCG (96 hs). Control (**C**) and G4/6^{prko} (**D**) ovaries from animals at day 9 of pregnancy. *, tubal occlusion; #, columnar epithelial cell hypertrophy. N=3

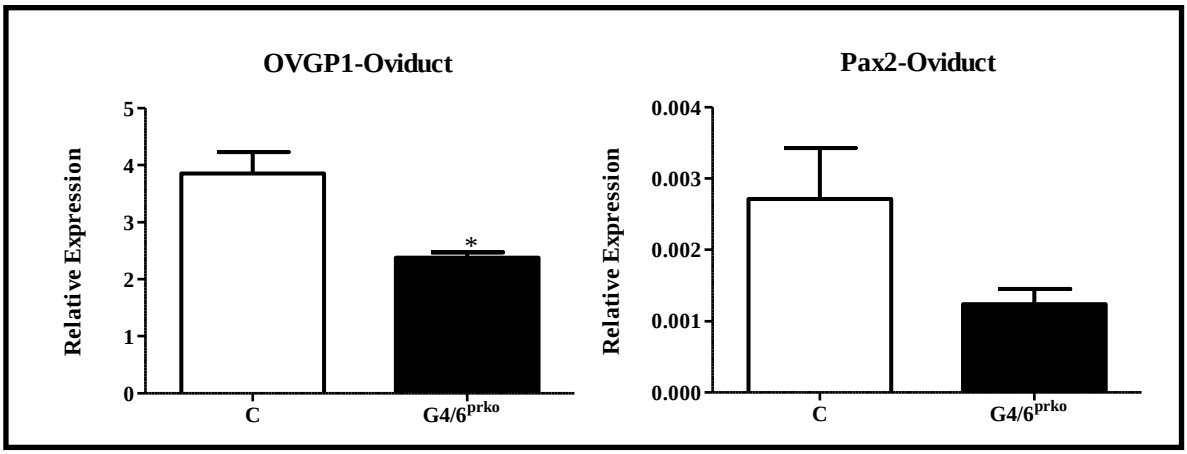


Figure 33. Altered Oviductal Gene Expression in GATA4/6^{prko} Animals

mRNA expression of oviductal genes from eCG/hCG (96 hs) control (C) and G4/6^{prko} animals. mRNA was isolated from whole oviducts. mRNA expression is relative to mouse ribosomal L19 (N≥4). *Columns* denote the average ± SEM. *, $P<0.05$.

downregulated in ovarian cancer. OVGP1 expression was significantly decreased in the G4/6^{prko} animals whereas Pax2 expression had a tendency to decrease in mutant animals, although this difference did not reach statistical significance. These results suggest that alterations in oviductal gene expression of OVGP1 and Pax2 may contribute to the G4/6^{prko} infertile phenotype.

In the G4/6^{prko} mice treated with eCG/hCG (96 hs), not only was Pax2 decreased in the oviduct at the mRNA level but it was also decreased at the protein level as shown by immunohistochemistry (Fig. 34). In Figure 34, we were also able to confirm the reduced expression of Pax2 (Fig. 27) in the ovaries of the G4/6^{prko} animals. These findings suggest that GATA factors regulate Pax2 in the oviduct and ovary. However, whether Pax2 is a direct target of GATA factors remains unknown.

There appears to be no significant alterations in the morphology of the uteri (Fig. 35A). However, there was a significant decrease in Pla2g4a expression in the uterus (Fig. 35B). Phospholipase A2, Group IVA (Pla2g4a) is an enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic molecules and is crucial for normal implantation to occur (157). In contrast, mitogen activated protein kinase kinase kinase 5 (Map3k5), which is involved in MAPK signaling and has been implicated in human receptivity for implantation (157), only had a tendency to be upregulated in the absence of GATA factors but this was not significant. These results suggest that the uterus is not as highly affected as the ovary or oviduct in the GATA4/6^{prko}. However, expression of GATA4 and GATA6 are mainly in the epithelial cells of the uterus and the mRNA samples also included the stroma. Thus, further experiments need to be done to look at how the loss of GATA factors in the epithelial cells of the uterus impact uterine function.

C. Discussion

GATA factors are highly expressed during folliculogenesis within the granulosa cells and also in the luteal cells of the corpus luteum. Previous experiments in which GATA factors were knocked down utilizing the Cyp19-Cre mouse (Chapter III) prevented examining the role of GATA factors in luteal

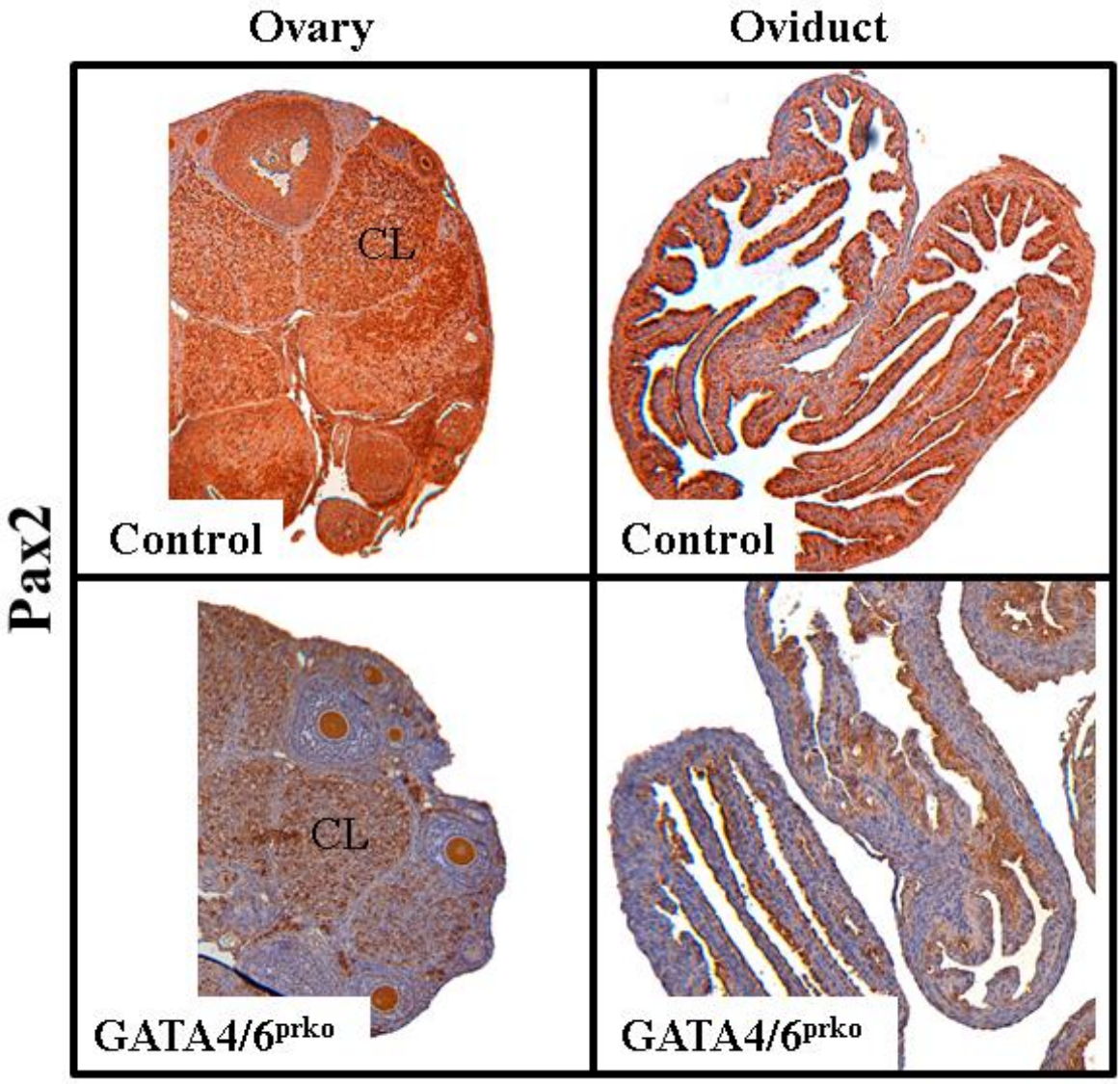


Figure 34. Decreased Pax2 Expression in GATA4/6^{prko}

Control and GATA4/6^{prko} ovaries (left) and oviducts (right) stained for Pax2 protein in animals treated with eCG/ hCG (96 hs). Representative pictures are shown. Brown = positive staining for Pax2. Blue = hematoxylin counterstain.

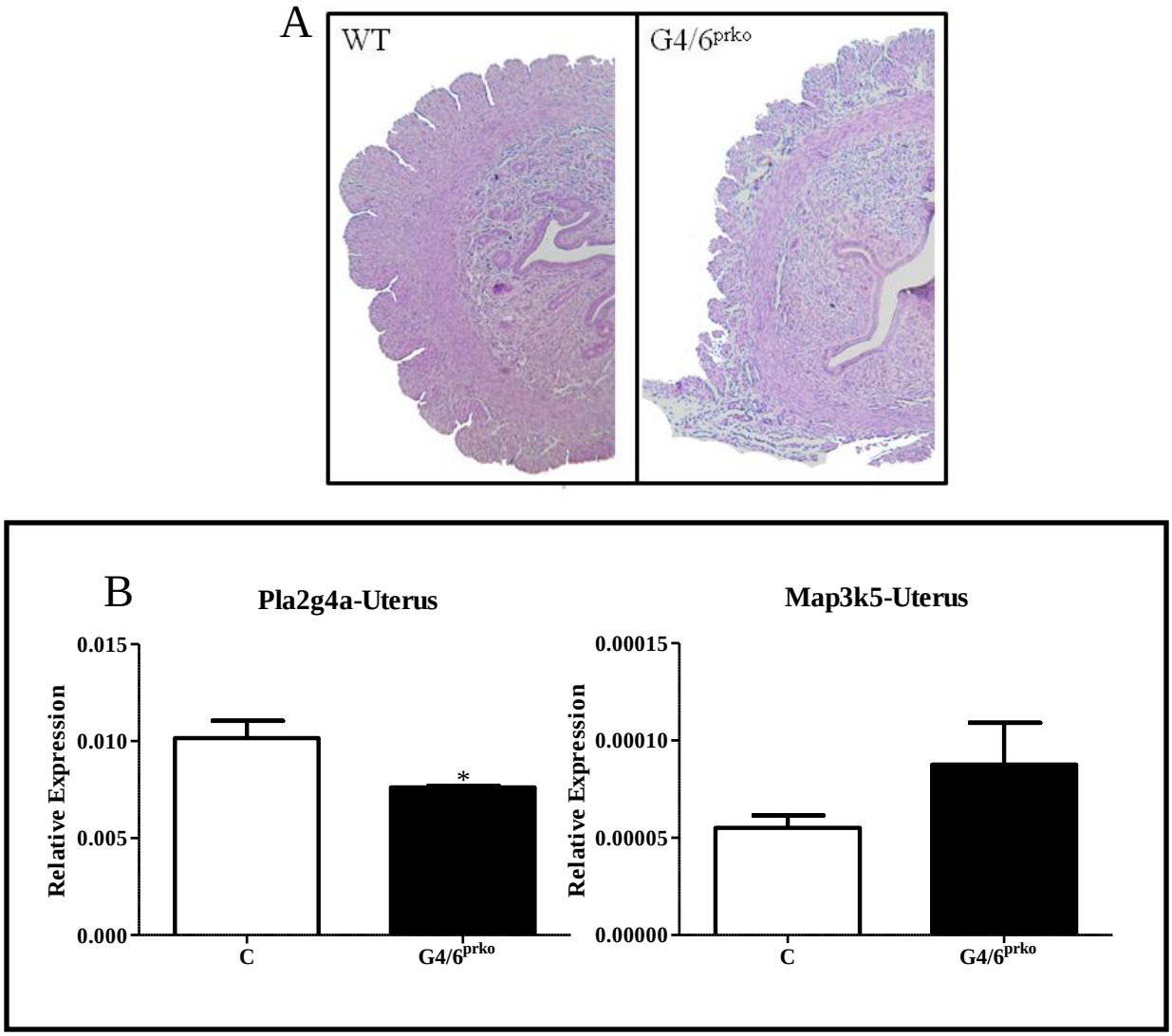


Figure 35. Altered Uterine Gene Expression in GATA4/6^{prko}

A) Hematoxylin and eosin staining of control and G4/6^{prko} uteri treated with eCG/hCG (96 hs). Representative pictures are shown. N=3. **B**) mRNA expression of uterine genes from eCG/ hCG-treated (96 hs) animals. mRNA was isolated from whole uteri. mRNA expression is relative to mouse ribosomal L19 (N≥3). Columns denote the average ± SEM. *, $P<0.05$

cells as corpora lutea did not form in these animals. Knowing that GATA factors could act on a diverse array of functional pathways, including those for steroidogenesis (Chapter III), we hypothesized that GATA factors impact corpora lutea function and female fertility. Using double GATA4 and GATA6 progesterone receptor specific conditional knockout animals, we documented that both factors are necessary for progesterone synthesis and female fertility.

Lack of progesterone synthesis and secretion from the ovary seems to be the major cause of infertility in GATA4/6^{prko} mice as progesterone administration restores implantation in some animals. These findings suggest that the lack of progesterone is not the only factor contributing to the infertility phenotype. It is possible that other functional pathways necessary for implantation have been impaired. There is decreased expression of Vcan in the ovaries of the GATA4/6^{prko}. Vcan is an extracellular matrix protein crucial for ovulation, and is expressed, not only in the ovary but also in the oviduct and uterus. Vcan is thought to be involved with cell migration, developing tissue pattern formation and potentially control trophoblast cell invasion within the uterus (158).

The knockout of the GATA factors in the oviduct and uterus are also likely contributing to the infertile phenotype of the GATA4/6^{prko}. OVGP1 secretion is increased within the secretory cells of the oviduct by estradiol and downregulated by progesterone (159). In contrast to this, OVGP1 was significantly downregulated in GATA4/6^{prko} animals despite that progesterone levels are low, suggesting that GATA factors influence this factor by another means than altering progesterone levels.

Much like the GATA family of transcription factors, the Pax family of transcription factors is important for development. Pax2 null mice die perinatally and lack oviducts, kidneys, ureters and have a defective central nervous system (160,161). Interestingly, Pax2 has been shown to regulate the expression of GATA3 during kidney development (162). In the oviduct, Pax2, GATA4 and GATA6 are highly expressed within the luminal epithelium. Pax2 downregulation is thought to be a marker for ovarian cancer (155). Interestingly, we observed cell hypertrophy in the oviducts of GATA4/6^{prko} animals, which

correlates with a decrease in Pax2 expression. The interaction between GATA factors and Pax factors within the oviduct is of great interest as it could provide new insights into the ovarian cancer field.

Phospholipase A2 enzymes are involved in the rate limiting step of prostaglandin synthesis but how these enzymes are regulated is still highly unknown (163). Prostaglandins can promote corpus luteum regression and parturition but they are typically inhibited during pregnancy so that progesterone synthesis is not interrupted. Mice with a null mutation for Pla2g4a have a delay in the onset of implantation, fewer implantation sites and smaller litters (164,165) which is of interest as implantation is defective in GATA4/6^{prko} females. However, this protein has been shown to be negatively correlated with progesterone concentrations (166), which we do not see in the GATA4/6^{prko} females. Similarly to GATA4 and 6, Pla2g4a is localized to the luminal epithelium of the uterus (166). Additionally, not only was Pla2g4a expression significantly decreased in the GATA4/6^{prko}, but its expression was also significantly decreased in the GATA4/6 granulosa cell specific knockout animals (Chapter IV), suggesting that this enzyme is regulated by GATA factors.

Collectively, these data show for the first time that GATA factors are essential for progesterone production *in vivo*. The steroidogenic enzymes necessary for luteal progesterone synthesis (Cyp11a1 and StAR) decrease significantly in the absence of GATA, which correlates with a reduction in plasma progesterone levels, but not with changes in corpora lutea structure. As the use of PR-Cre animals does not just knock down these factors in the ovary, further experiments are needed to determine the roles of GATA factors within the oviduct and uterus and to determine if the alterations in these tissues is a result of impaired progesterone production or a direct effect of the loss of GATA factors.

VI. CONCLUSIONS AND FUTURE DIRECTIONS

Due to the embryonic lethality of GATA4 and GATA6 knockout animals (58,73), the functional role that these factors have in the ovary was unknown. Here, we show for the first time that GATA factors are crucial for normal preovulatory follicle development and female fertility. This essential role of GATA4 in the ovary seems to be mediated at least in part by the stimulation of the FSHR; thus GATA4 regulates FSH signaling. However, we showed that GATA not only regulates genes through the FSH signaling pathway but also directly influences gene expression of a number of genes including Cyp19a1 and Papp-a. It will be important to confirm the direct interaction of GATA factors to the promoters of genes found to be regulated in the absence of GATA factors, especially those that have GATA binding sites, such as Vcan and Inhα.

Interestingly, women carrying an inactivating mutation of the *Fshr* gene express little GATA4 protein in their ovaries (167). As expected, the ovaries of these women lack significant follicular development. In mice, eCG and FSH enhance the expression of GATA4 and GATA6 transcripts (61,71). Moreover, we have previously demonstrated that FSH signaling leads to the phosphorylation of GATA4 on serine 105 (71), a modification that is known to increase the transcriptional activity of GATA4 (168). This evidence, along with the reduction in *Fshr* expression after loss of GATA function, suggests the presence of a positive feedback system between FSH and GATA in ovarian granulosa cells. We propose that this positive feedback is important for the differentiation of granulosa cells and the rapid growth of preantral and early antral follicles to the preovulatory stage. Low FSHR expression accounts for the poor ovarian response to gonadotropin stimulation found in approximately 25% of women undergoing *in vitro* fertilization (169-171). There is no evidence suggesting that GATA factors could be involved in the regulation of FSHR expression in humans. It would be of great interest, then, to examine whether low levels of FSHR in poor-responding women correlate with defects in GATA4 expression and/or activity.

In humans, GATA4 inactivating mutations in the gonads and single nucleotide polymorphisms (SNPs) such as G93A, L403M, L432S, and A263G have been described (172-174). The relationship between these mutations and female fertility has not been assessed. However, since our results demonstrated for the first time a crucial role for GATA4 on FSHR expression in granulosa cells, it is possible to speculate that lack of a normal response to FSH in infertile women could result from the presence of SNPs in the *GATA4* gene, altering its activity in the ovary. It would therefore be important to perform clinical studies to determine whether GATA4 SNPs correlate with a decrease of the response of human granulosa cells to FSH. In addition, this information would help on finding better IVF stimulation protocols for patients carrying GATA4 mutations.

The microarray analysis of animals lacking GATA in granulosa cells demonstrated that GATA4 appeared to have a more significant impact compared to GATA6 on granulosa cell function. While GATA4 and GATA6 have 85% homology at their DNA binding domains (57), suggesting they can bind and regulate the same genes, the slight differences within their DNA binding domains could alter how these factors binding the W-GATA-R binding motif. GATA factors have been shown to differentially bind the GATA binding motif and show preference for certain sequences as well as stronger binding to certain sequences (175). Differences between the transcriptional activation domains of GATA4 and GATA6 can also impact protein-protein interactions between the GATA factors and their cofactors, which can create differences in the intensity of GATA factors binding to their binding motif to activate genes (70). Another reason GATA4 but not GATA6 has more importance within granulosa cells could result from its activation by posttranslational modifications. GATA4 has phosphorylation sites at ser105 and ser261 (71) while GATA6 has phosphorylation sites at ser120 and ser192 (176). The phosphorylation of these sites could be differentially regulated in granulosa cells and lead to a higher activation of GATA4 over GATA6. The differences between GATA4 and GATA6 binding to the promoters of regulated genes in granulosa cells remains to be determined.

There are significant differences between the loss of GATA4 and GATA6 in granulosa cells compared to the loss of GATA4 and GATA6 in luteal cells. In granulosa cells, GATA4 had more impact on gene regulation of granulosa cell function (i.e. FSHR and aromatase) than GATA6. However, in luteal cells GATA4 and GATA6 both appear to be equally important. Unlike in the GATA6^{gcko} which did not have a phenotype, the GATA6^{prko} resulted in subfertility, suggesting that GATA6 might have a more significant role in the ovary after ovulation. It appears that the functional roles of the GATA factors change during the course of folliculogenesis. This could result from alterations in cofactor expression between the two cell types, which would alter target gene expression or alterations in the activation of GATA6. Microarrays on the progesterone responsive tissue knockouts should be done as this data could then be compared to the data we obtained in GATA^{gcko} animals to determine the changes in the functional roles of the GATA factors during folliculogenesis and corpus luteum formation. This would give insight into what genes are consistently regulated by the GATA factors in granulosa and luteal cells and which genes are differentially affected in follicles and corpora lutea. The present work focused only on the double knockout as it had the most significant phenotype of infertility. It will be important to determine the phenotypes of the individual GATA knockout animals as they each have a subfertile phenotype. This would also aide in giving insight into the specific roles GATA4 and GATA6 have in luteal cells.

In knocking down the GATA factors within the granulosa cells of the preovulatory follicles, we show that these factors are crucial for luteal progesterone synthesis. However, as progesterone levels did not decrease initially after ovulation (17 hs after hCG) in the GATA4/6^{prko} but significantly decreased 96 hs after hCG, questions arise of when the GATA factors become crucial for CL function. A time course experiment where plasma samples and corpora lutea are collected from animals 24, 48, and 72 hs after hCG treatment should be performed to fully characterize the effect of the lack of GATA factors during corpus luteum formation. The absence of implantation sites in the GATA4/6^{prko}, which was recovered in one of two animals, is also intriguing. These results suggest that the loss of progesterone partially

contributes to the infertility phenotype of these animals. However, this experiment should be repeated and a higher number of animals included in order to reach a final conclusion.

Although we show a significant decrease in progesterone levels within our G4/6^{prko} females, we also show that corpora lutea are able to form in these animals. This is in despite of the low expression of prolactin receptor and luteinizing hormone receptor after treatment with hCG for 96 hs, although this data should be cautiously taken into account as we have not determine the protein levels of these receptors.. It is also possible that the CL have impaired luteolysis and this is why we see CL in the ovaries of our G4/6^{prko} females. It was also shown that the CL do not have an increase in apoptosis and have normal expression of the vascularization promoting factor, VEGF. However, decreased progesterone levels could also occur if the receptor for VEGF were decreased or if vascularization were not occurring normally. Experiments looking into the impact of GATA factors on luteal cell vascularization should be further assessed. Extracellular matrix (ECM) remodeling and reorganization is required for the formation of new blood vessels (177). The novel observation that several genes involved in the regulation of ECM are affect by the lack of GATA4 and GATA6 expression in follicles suggest that knockdown of GATA factor during luteinization may impair the neovascularization of newly formed corpora lutea.

Lastly, even though luteal production of progesterone is reduced in the absence of GATA factors, it is also possible that whatever progesterone being made is metabolized to an inactive state. The catabolism of progesterone in GATA^{prko} mice should be assessed by quantifying the expression of the enzyme 20α hydroxysteroid dehydrogenase, which is the main enzyme involved in the inactivation and catabolism of progesterone in the rodent CL (10).

The roles that GATA factors have in the oviduct and uterus remain unknown. Thus far, we know some key fertility-related genes within the oviduct (e.g. OVGP1) and uterus (e.g. Pla2g4a) are regulated by GATA. However, how these genes contribute to the phenotype observed is not known. Moreover, it is unknown whether the genes affected in the oviducts and uteri of GATA4/6^{prko} are direct targets of

GATA or whether they are affected by the decrease in progesterone. Of interest is that the knockout of GATA factors resulted in abnormal morphology of the oviduct. However, whether the loss of GATA factors affected the ciliated cells or the secretory cells of the oviduct is still unknown. GATA factors influence both proliferation and apoptotic pathways in the ovary (Chapter III) as well as the gut (178,179) and heart (180,181); it is possible that they influence these pathways within the oviduct as well. This is of particular interest as decreased GATA factor expression has been linked to ovarian cancer (182,183). Additionally, the decreased expression of Pax2 has also been found in ovarian cancers (184). Here we show that loss of GATA factors downregulates Pax2 in both the ovary and oviduct. Thus, learning about GATA regulation of Pax2 could be of interest to the ovarian cancer field.

In conclusion, the results presented in this thesis work revealed for the first time a crucial role for GATA4 and GATA6 during the folliculogenesis and luteinization processes. These novel findings contribute significantly to our understanding of follicle maturation and provide the background for future studies aimed to determine the molecular pathways involved in the regulation of key steroidogenic, ECM, and signaling genes by GATA factors in the ovary.

APPENDICES

APPENDIX A

TABLE III. GENES DIFFERENTIALLY EXPRESSED AMONG WT AND GATA4^{GCKO}

224 genes were significant at P<0.01 with a fold change of 2 or more.

Downregulated Genes

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4^{gcko}				
Comp	12845	491.63	30.8	-15.96	1.00E-07	0.00018	cartilage oligomeric matrix protein
Cyp17a1	13074	737.04	83.76	-8.80	2.30E-04	0.0238	cytochrome P450, family 17, subfamily a, polypeptide 1
Gabrb2	14401	324.67	41.5	-7.82	1.00E-07	1E-07	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2
Ctsc	13032	183.52	30.28	-6.06	1.00E-07	1E-07	cathepsin C
Grem1	23892	332.4	60.04	-5.54	1.60E-06	0.00125	gremlin 1
Tnfsf11	21943	313.95	57.34	-5.48	1.10E-06	0.00103	tumor necrosis factor (ligand) superfamily, member 11
Adh1	11522	488.29	92.02	-5.31	1.70E-05	0.00553	alcohol dehydrogenase 1 (class I)

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Masp1	17174	549.15	108.88	-5.04	8.00E-07	0.00089	mannan-binding lectin serine peptidase 1
Cd34	12490	371.98	77.88	-4.78	8.00E-07	0.00089	CD34 antigen
Gstm6	14867	120.79	25.5	-4.74	2.23E-03	0.0671	glutathione S-transferase, mu 6
Lypd6	320343	116.1	25.7	-4.52	2.82E-04	0.0268	LY6/PLAUR domain containing 6
Plp1	18823	187.76	43.78	-4.29	4.10E-06	0.00243	proteolipid protein (myelin) 1
Cdh2	12558	440.14	105.1	-4.19	9.80E-06	0.00409	cadherin 2
Rimklb	108653	682.67	166.15	-4.11	1.30E-06	0.00107	ribosomal modification protein rimK-like family member B
Defb19	246700	233.75	57.12	-4.09	1.00E-07	1E-07	defensin beta 19
Rassf2	215653	216.87	53.72	-4.04	3.60E-06	0.00237	Ras association (RalGDS/AF-6) domain family member 2
Zpld1	239852	112.39	28	-4.01	1.27E-05	0.00484	zona pellucida like domain containing 1

APPENDIX A (continued)

		Mean of Intensities					
Symbol	**EntrezID**	**WT**	**GATA4^{gcko}**	**Fold-change**	**p-value**	**FDR**	**Name**
Nup62cl	279706	128.38	32.28	-3.98	3.70E-06	0.00238	nucleoporin 62 C-terminal like
Gabra1	14394	120.57	31.49	-3.83	1.20E-06	0.00105	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1
Mro	71263	753.11	204.52	-3.68	1.00E-07	0.00018	maestro
Gsta4	14860	659.42	182.92	-3.60	5.00E-07	0.00069	glutathione S-transferase, alpha 4
Tom1l1	71943	965.85	268.62	-3.60	5.60E-06	0.00279	target of myb1-like 1 (chicken)
Lect1	16840	753.79	213.68	-3.53	8.40E-06	0.00362	leukocyte cell derived chemotaxin 1
1110032F04Rik	68725	145.18	41.25	-3.52	3.84E-04	0.0299	RIKEN cDNA 1110032F04 gene
Rgs13	246709	162.85	47.97	-3.39	5.12E-05	0.0107	regulator of G-protein signaling 13
Arrdc4	66412	117.14	35.55	-3.30	1.00E-07	1E-07	arrestin domain containing 4
Etohi1	626848	232.06	70.57	-3.29	3.27E-03	0.079	ethanol induced 1
Mapkbp1	26390	228.11	70.3	-3.24	3.05E-05	0.00774	mitogen-activated protein kinase binding protein 1
Plxnc1	54712	1571.83	499.66	-3.15	1.30E-06	0.00107	plexin C1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Fdx1	14148	325.95	105.62	-3.09	6.63E-04	0.0377	ferredoxin 1
4933409K07Rik	108816	147.97	47.99	-3.08	2.00E-03	0.064	RIKEN cDNA 4933409K07 gene
Tox	252838	102	33.6	-3.04	3.00E-07	0.00048	thymocyte selection-associated high mobility group box
St3gal1	20442	140.38	46.62	-3.01	3.33E-03	0.0798	ST3 beta-galactoside alpha-2,3-sialyltransferase 1
Tmeff2	56363	89.39	29.84	-3.00	5.88E-05	0.0118	transmembrane protein with EGF-like and two follistatin-like domains 2
Susd4	96935	177.94	60.86	-2.92	7.00E-07	0.00088	sushi domain containing 4
Gm3893	100042539	136.08	46.8	-2.91	2.40E-03	0.0696	predicted gene 3893
Lrrtm3	216028	153.42	53.11	-2.89	1.38E-04	0.0192	leucine rich repeat transmembrane neuronal 3
Fshr	14309	720.69	251.31	-2.87	9.00E-07	0.00093	follicle stimulating hormone receptor
Krtap16-4	170654	86.41	30.14	-2.87	8.77E-04	0.0431	keratin associated protein 16-4
Gm10220	434689	224.23	80.27	-2.79	6.38E-04	0.0367	predicted gene 10220

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Sema3g	218877	165.87	59.44	-2.79	4.70E-06	0.00243	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G
Slxl1	75140	115.67	41.54	-2.78	5.24E-04	0.0334	Slx-like 1
5031410I06Rik	381622	259	93.12	-2.78	4.53E-04	0.0315	RIKEN cDNA 5031410I06 gene
Rrs1	59014	256.44	92.41	-2.78	3.63E-04	0.0291	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)
Ccbl2	229905	157.85	57.28	-2.76	1.10E-06	0.00103	cysteine conjugate-beta lyase 2
Rem1	19700	78.35	28.82	-2.72	4.00E-07	0.00058	rad and gem related GTP binding protein 1
Ces2g	72361	77.72	28.66	-2.71	1.14E-05	0.00458	carboxylesterase 2G
Gm10471	100039045	172.79	63.76	-2.71	1.06E-03	0.0479	predicted gene 10471
Satb2	212712	82.6	30.63	-2.70	2.07E-05	0.00576	special AT-rich sequence binding protein 2
Chst15	77590	90.81	33.8	-2.69	4.98E-04	0.0329	carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4^{gcko}				
Cyp19a1	13075	1737.67	648.95	-2.68	1.35E-03	0.0524	cytochrome P450, family 19, subfamily a, polypeptide 1
Mup2	17841	113.68	42.88	-2.65	7.37E-04	0.0395	major urinary protein 2
Amy2a5	109959	107.29	40.73	-2.63	3.08E-03	0.0769	amylase 2a5
Cacna1d	12289	74.59	28.39	-2.63	1.54E-05	0.00531	calcium channel, voltage-dependent, L type, alpha 1D subunit
Fn1	14268	265.39	101.92	-2.60	9.42E-03	0.139	fibronectin 1
Chst1	76969	78.58	30.81	-2.55	3.64E-04	0.0291	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
Vmn2r43	381838	100.27	39.38	-2.55	6.00E-03	0.107	vomeronasal 2, receptor 43
Gm14354	74851	122.39	48.57	-2.52	3.10E-03	0.077	predicted gene 14354
Alms1	236266	183.67	73.8	-2.49	2.80E-06	0.00188	Alstrom syndrome 1 homolog (human)
Pcx	18563	211.79	86.45	-2.45	4.70E-06	0.00243	pyruvate carboxylase

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Ralgapa2	241694	253.11	103.71	-2.44	1.50E-06	0.00121	Ral GTPase activating protein, alpha subunit 2 (catalytic)
Rhox8	434768	95.66	39.6	-2.42	2.04E-05	0.00576	reproductive homeobox 8
Prrg1	546336	64.99	27.07	-2.40	3.19E-04	0.0282	proline rich Gla (G-carboxyglutamic acid) 1
D14Ertd449e	66039	277.36	115.61	-2.40	2.09E-05	0.00576	DNA segment, Chr 14, ERATO Doi 449, expressed
1700084J12Rik	73486	80.99	34.03	-2.38	6.80E-05	0.0125	ribosomal protein L7-like 1 pseudogene
C130026I21Rik	620078	92.39	39.17	-2.36	1.89E-03	0.0623	RIKEN cDNA C130026I21 gene
Ott	18422	80.41	34.19	-2.35	3.11E-03	0.077	ovary testis transcribed
Agrn	11603	120.22	51.14	-2.35	1.12E-04	0.017	agrin
Igk	243469	117.33	50.11	-2.34	1.73E-03	0.0595	immunoglobulin kappa chain complex
Gm5168	382275	93.58	40.02	-2.34	1.17E-03	0.0487	predicted gene 5168
Aldh1a1	11668	1182.77	505.94	-2.34	1.84E-05	0.00573	aldehyde dehydrogenase family 1, subfamily A1
Npr1	18160	88.25	37.86	-2.33	1.38E-03	0.0529	natriuretic peptide receptor 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Vmn2r34	100042636	82.92	35.68	-2.32	8.16E-03	0.128	vomeronasal 2, receptor 34
Mup7	100041658	110.61	47.61	-2.32	1.63E-03	0.0578	major urinary protein 7
Enpep	13809	72.51	31.22	-2.32	3.99E-04	0.0307	glutamyl aminopeptidase
Mlh1	17350	78.19	33.69	-2.32	5.10E-05	0.0107	mutL homolog 1 (E. coli)
Gm5589	434166	93.78	40.41	-2.32	8.46E-03	0.131	predicted gene 5589
Robo2	268902	105.17	45.51	-2.31	1.38E-03	0.053	roundabout homolog 2 (Drosophila)
Olfr1383	404337	91.46	39.62	-2.31	4.18E-03	0.0899	olfactory receptor 1383
Gm6682	626534	548.87	238.54	-2.30	2.10E-06	0.00156	predicted gene 6682
Il28a	330496	190.38	83.13	-2.29	1.06E-03	0.0479	interleukin 28A
Adamts12	239337	83.82	36.74	-2.28	4.47E-05	0.00988	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 12
Mapre2	212307	436.71	191.6	-2.28	2.40E-05	0.00643	microtubule-associated protein, RP/EB family, member 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Dad1	13135	651.42	289.53	-2.25	7.30E-04	0.0394	defender against cell death 1
Slc7a8	50934	902.23	401.93	-2.24	1.31E-05	0.00492	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
Ryr2	20191	65.56	29.33	-2.24	1.09E-05	0.00444	ryanodine receptor 2, cardiac
Elovl2	54326	70.45	31.54	-2.23	2.03E-05	0.00576	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2
Abpz	233090	66.78	29.97	-2.23	5.51E-04	0.0343	androgen binding protein zeta
D0H4S114	27528	385.92	173.9	-2.22	4.60E-06	0.00243	DNA segment, human D4S114
Vmn1r79	100042437	96.91	43.97	-2.20	1.56E-03	0.0566	vomeronasal 1 receptor 79
Tmsb15l	399591	60.49	27.51	-2.20	2.50E-06	0.00181	thymosin beta 15b like
Ndn	17984	118.7	53.99	-2.20	7.63E-03	0.123	necdin
Luzp4	434865	74.46	33.9	-2.20	2.38E-03	0.0695	leucine zipper protein 4
Gm5114	330513	64.04	29.38	-2.18	4.52E-04	0.0315	predicted gene 5114
Parm1	231440	259.67	119.24	-2.18	9.47E-04	0.045	prostate androgen-regulated mucin-like protein 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Vmn2r28	665255	60.79	27.93	-2.18	5.88E-04	0.0355	vomeronasal 2, receptor 28
Aplnr	23796	74.92	34.47	-2.17	6.20E-03	0.109	apelin receptor
Myo18b	74376	209.25	96.63	-2.17	3.82E-05	0.00899	myosin XVIIIb
Mir598	100124452	219.45	101.56	-2.16	1.45E-04	0.0199	microRNA 598
Bves	23828	97.84	45.45	-2.15	4.38E-04	0.0314	blood vessel epicardial substance
Xlr5a	574438	72.09	33.56	-2.15	2.73E-04	0.0265	X-linked lymphocyte-regulated 5A
Dock5	68813	384.4	179.58	-2.14	1.22E-05	0.00484	dedicator of cytokinesis 5
Olfr460	258381	65.7	30.7	-2.14	9.36E-03	0.139	olfactory receptor 460
Ccdc68	381175	82.5	38.57	-2.14	2.41E-03	0.0696	coiled-coil domain containing 68
Vmn2r122	22308	65.28	30.58	-2.13	3.38E-04	0.0286	vomeronasal 2, receptor, 122
P2ry13	74191	58.01	27.2	-2.13	4.26E-03	0.0902	purinergic receptor P2Y, G-protein coupled 13
Hcn1	15165	292.56	137.34	-2.13	6.03E-05	0.0118	hyperpolarization-activated, cyclic nucleotide-gated K+ 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Defa21	66298	61.46	28.9	-2.13	2.52E-03	0.0703	defensin, alpha, 21
Tmem178	68027	94.83	44.63	-2.12	3.58E-04	0.0291	transmembrane protein 178
Mup11	100039028	69.89	32.91	-2.12	1.28E-03	0.0511	major urinary protein 11
Prickle2	243548	56.65	26.78	-2.12	5.23E-05	0.0108	prickle homolog 2 (Drosophila)
Hunk	26559	166.39	78.87	-2.11	4.60E-06	0.00243	hormonally upregulated Neu-associated kinase
Olfr1034	258216	81.6	38.71	-2.11	3.68E-05	0.00873	olfactory receptor 1034
Cebpa	12606	107.65	51.26	-2.10	3.95E-04	0.0305	CCAAT/enhancer binding protein (C/EBP), alpha
Rab11fip1	75767	63.53	30.27	-2.10	4.58E-05	0.01	RAB11 family interacting protein 1 (class I)
Pdlim2	213019	83.92	40.03	-2.10	7.00E-06	0.00322	PDZ and LIM domain 2
Cyb5	109672	2261.3	1085.54	-2.08	4.92E-05	0.0107	cytochrome b-5
Vcan	13003	1524.26	731.81	-2.08	5.20E-04	0.0334	versican
Olfm1	56177	77.69	37.32	-2.08	8.00E-06	0.00362	olfactomedin 1
Iqgap2	544963	112.24	54.07	-2.08	3.61E-04	0.0291	IQ motif containing GTPase activating protein 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
BC056474	414077	454.63	219.9	-2.07	2.05E-04	0.0229	cDNA sequence BC056474
Cdk18	18557	66	32	-2.06	8.69E-04	0.0428	cyclin-dependent kinase 18
Lsr	54135	70.66	34.29	-2.06	9.13E-03	0.137	lipolysis stimulated lipoprotein receptor
Olfr1200	257887	63.67	30.9	-2.06	1.86E-03	0.0617	olfactory receptor 1200
Igsf3	78908	238.2	116.27	-2.05	1.26E-05	0.00484	immunoglobulin superfamily, member 3
Ctsh	13036	210.16	102.81	-2.04	2.31E-03	0.0682	cathepsin H
Gm15107	434864	62.49	30.6	-2.04	6.33E-03	0.111	predicted gene 15107
Prlr	19116	575.55	282	-2.04	5.03E-04	0.0329	prolactin receptor
Adamts1	11504	195.47	95.96	-2.04	1.75E-05	0.00557	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1
Gm5622	434459	88.96	43.69	-2.04	3.05E-03	0.0768	predicted gene 5622
Vmn1r221	100312485	81.93	40.24	-2.04	2.19E-03	0.0669	vomeronasal 1 receptor 221
Gpc6	23888	66.59	32.85	-2.03	1.11E-04	0.017	glypican 6

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Tuba1a	22142	822.9	406.47	-2.02	9.48E-04	0.045	tubulin, alpha 1A
Armcx2	67416	185.82	91.82	-2.02	4.03E-04	0.0308	armadillo repeat containing, X-linked 2
Mfge8	17304	425.64	210.37	-2.02	1.36E-05	0.00497	milk fat globule-EGF factor 8 protein
Mid1ip1	68041	626.43	310.69	-2.02	5.70E-06	0.0028	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish))
Fam196a	627214	104.3	51.77	-2.01	9.24E-05	0.0152	family with sequence similarity 196, member A
Vmn2r85	623734	65.66	32.62	-2.01	2.58E-03	0.071	vomeronasal 2, receptor 85
Scoc	56367	80.34	39.92	-2.01	9.33E-03	0.139	short coiled-coil protein
Fam162b	77296	139.99	69.66	-2.01	1.00E-03	0.0463	family with sequence similarity 162, member B
Slc12a7	20499	353.05	175.79	-2.01	2.40E-04	0.0243	solute carrier family 12, member 7
Snord7	100302731	157.58	78.54	-2.01	6.67E-04	0.0377	small nucleolar RNA, C/D box 7
Avpi1	69534	59.36	29.63	-2.00	3.06E-03	0.0769	arginine vasopressin-induced 1

APPENDIX A (continued)

Upregulated Genes

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4^{gcko}				
Gm13691	668119	179.06	1444.87	8.07	1.00E-07	1E-07	CWC22 spliceosome-associated protein homolog pseudogene
Bcan	12032	31.37	200.05	6.38	2.80E-06	0.00188	brevican
Cyp1b1	13078	282.01	1326.69	4.70	4.00E-07	0.00058	cytochrome P450, family 1, subfamily b, polypeptide 1
Mapk10	26414	49.12	229.15	4.67	1.00E-07	0.00018	mitogen-activated protein kinase 10
Itih2	16425	33.22	144.1	4.34	2.00E-07	0.00034	inter-alpha trypsin inhibitor, heavy chain 2
Slc18a2	214084	200.64	826.01	4.12	4.30E-06	0.00243	solute carrier family 18 (vesicular monoamine), member 2
2010110P09Rik	70261	28.25	115.83	4.10	2.00E-06	0.00152	RIKEN cDNA 2010110P09 gene
Cwc22	80744	49.17	185.56	3.77	2.19E-05	0.00598	CWC22 spliceosome-associated protein homolog (S. cerevisiae)
Adck3	67426	76.17	271.82	3.57	5.10E-06	0.00259	aarF domain containing kinase 3

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Nos2	18126	51.1	180.16	3.53	3.90E-06	0.00243	nitric oxide synthase 2, inducible
Gadd45b	17873	51.21	172.08	3.36	6.70E-06	0.00313	growth arrest and DNA-damage-inducible 45 beta
Limch1	77569	40.35	130.09	3.22	6.30E-06	0.00299	LIM and calponin homology domains 1
Nupr1	56312	90.12	265.93	2.95	1.00E-07	1E-07	nuclear protein 1
Hpgd	15446	315.89	900.08	2.85	6.14E-05	0.0118	hydroxyprostaglandin dehydrogenase 15 (NAD)
Leprel1	210530	69.58	196.11	2.82	4.52E-04	0.0315	leprecan-like 1
Fam110c	104943	30.55	84.72	2.77	4.00E-06	0.00243	family with sequence similarity 110, member C
Gadd45a	13197	56.91	154.45	2.71	1.54E-05	0.00531	growth arrest and DNA-damage-inducible 45 alpha
Sytl2	83671	39.54	106.72	2.70	8.00E-07	0.00089	synaptotagmin-like 2
Gjc3	118446	28.92	77.86	2.69	1.51E-03	0.0558	gap junction protein, gamma 3
Rarres2	71660	69.32	184.93	2.67	2.41E-03	0.0696	retinoic acid receptor responder (tazarotene induced) 2
Emx2	13797	48.69	129.08	2.65	1.03E-04	0.0162	empty spiracles homolog 2 (Drosophila)

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Ank2	109676	37.09	96.69	2.61	3.53E-05	0.00851	ankyrin 2, brain
Pik3ip1	216505	65.81	171.16	2.60	8.50E-06	0.00362	phosphoinositide-3-kinase interacting protein 1
Gpr98	110789	55.99	144.97	2.59	1.61E-05	0.00539	G protein-coupled receptor 98
Mboat1	218121	107.93	274.91	2.55	1.19E-04	0.0174	membrane bound O-acyltransferase domain containing 1
E330013P04Rik	107376	37.43	94.94	2.54	4.07E-04	0.0308	RIKEN cDNA E330013P04 gene
Eya4	14051	33.94	85.95	2.53	1.73E-04	0.0215	eyes absent 4 homolog (Drosophila)
Gem	14579	49.33	123.83	2.51	2.75E-04	0.0265	GTP binding protein (gene overexpressed in skeletal muscle)
Pcsk6	18553	57.16	142.76	2.50	8.35E-05	0.0147	proprotein convertase subtilisin/kexin type 6
Grin2c	14813	44.12	107.71	2.44	9.00E-07	0.00093	glutamate receptor, ionotropic, NMDA2C (epsilon 3)
Kcnma1	16531	94.72	230.61	2.43	5.47E-05	0.0111	potassium large conductance calcium-activated channel, subfamily M, alpha member 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Nfib	18028	56.69	137.12	2.42	2.17E-04	0.0232	nuclear factor I/B
Sgpp2	433323	57.34	137.15	2.39	1.99E-05	0.00576	sphingosine-1-phosphate phosphotase 2
Kcnk1	16525	75.98	181.37	2.39	1.75E-03	0.0599	potassium channel, subfamily K, member 1
Adamts2	216725	265.13	632.24	2.38	2.09E-05	0.00576	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2
Tgfbr3	21814	130.72	311.53	2.38	4.05E-04	0.0308	transforming growth factor, beta receptor III
Klhl31	244923	69.71	160.96	2.31	3.24E-03	0.0786	kelch-like 31 (Drosophila)
Gpm6b	14758	116.64	268.76	2.30	3.30E-05	0.00819	glycoprotein m6b
Polr3g	67486	63.63	145.76	2.29	2.94E-04	0.0272	polymerase (RNA) III (DNA directed) polypeptide G
Sel1l3	231238	119.46	273.34	2.29	4.40E-06	0.00243	sel-1 suppressor of lin-12-like 3 (C. elegans)
Kcnq5	226922	112.6	257.34	2.29	6.63E-05	0.0124	potassium voltage-gated channel, subfamily Q, member 5
Angptl1	72713	72.66	165.65	2.28	3.22E-04	0.0282	angiopoietin-like 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Kazald1	107250	32.34	73.59	2.28	1.13E-04	0.0171	Kazal-type serine peptidase inhibitor domain 1
Kcnip3	56461	68.37	155.26	2.27	2.10E-04	0.0229	Kv channel interacting protein 3, calsenilin
Cd200	17470	75.04	168.65	2.25	6.70E-03	0.114	CD200 antigen
Gyltl1b	228366	221.86	497.23	2.24	4.60E-06	0.00243	glycosyltransferase-like 1B
Mycn	18109	52.35	117.25	2.24	1.78E-05	0.0056	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
AB041803	232685	63.22	140.87	2.23	1.30E-04	0.0185	cDNA sequence AB041803
Ssbp2	66970	124.13	273.82	2.21	6.26E-05	0.0119	single-stranded DNA binding protein 2
Nlrc5	434341	53.81	118.23	2.20	1.50E-05	0.00529	NLR family, CARD domain containing 5
Cnnm1	83674	107.42	235.9	2.20	2.21E-04	0.0235	cyclin M1
C130074G19Rik	226777	51.79	113.06	2.18	6.09E-05	0.0118	RIKEN cDNA C130074G19 gene
Bpifb5	228802	33.99	73.86	2.17	3.28E-04	0.0285	BPI fold containing family B, member 5
Plbd1	66857	234.26	507.83	2.17	1.11E-04	0.017	phospholipase B domain containing 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Sema4d	20354	45.45	97.67	2.15	5.90E-06	0.00285	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D
Maml2	270118	68.07	146.12	2.15	6.00E-04	0.0356	mastermind like 2 (Drosophila)
Myo1e	71602	276.85	585.92	2.12	6.87E-05	0.0125	myosin IE
Mblac2	72852	97.31	205.59	2.11	1.59E-04	0.0205	metallo-beta-lactamase domain containing 2
Sorbs2	234214	64.2	135.24	2.11	2.06E-03	0.0653	sorbin and SH3 domain containing 2
Rbms3	207181	57.48	120.33	2.09	5.26E-05	0.0108	RNA binding motif, single stranded interacting protein
Itga9	104099	229.9	480.78	2.09	3.03E-04	0.0274	integrin alpha 9
Igfbp2	16008	85.72	178.87	2.09	5.44E-04	0.034	insulin-like growth factor binding protein 2
Spon1	233744	61.99	129.34	2.09	1.17E-03	0.0487	spondin 1, (f-spondin) extracellular matrix protein
Fam78a	241303	129.38	269.78	2.09	2.85E-04	0.0269	family with sequence similarity 78, member A
Mkx	210719	153.61	319.75	2.08	1.56E-04	0.0204	mohawk homeobox

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	$GATA4^{gcko}$				
Myc	17869	191.44	398.45	2.08	1.69E-03	0.0587	myelocytomatosis oncogene
Sord	20322	93.99	195.15	2.08	1.91E-04	0.0226	sorbitol dehydrogenase
Sox18	20672	85.85	177.61	2.07	3.14E-04	0.0282	SRY-box containing gene 18
Fbn2	14119	221.71	457.77	2.06	2.94E-04	0.0272	fibrillin 2
Rhobtb1	69288	139.21	287.13	2.06	1.20E-03	0.0493	Rho-related BTB domain containing 1
Foxp2	114142	213.82	439.98	2.06	3.53E-04	0.0291	forkhead box P2
Fam171b	241520	328.98	676.38	2.06	1.31E-03	0.0514	family with sequence similarity 171, member B
Tgfb3	21809	108.03	221.88	2.05	5.06E-05	0.0107	transforming growth factor, beta 3
Synm	233335	53.06	108.92	2.05	8.50E-04	0.0423	synemin, intermediate filament protein
Cxx1c	72865	92.61	188.13	2.03	5.13E-03	0.0997	CAAX box 1 homolog C (human)
Bche	12038	112.93	228.74	2.03	4.09E-05	0.00934	butyrylcholinesterase

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4^{gcko}				
Fam46a	212943	89.17	179.16	2.01	3.03E-04	0.0274	family with sequence similarity 46, member A
Ano4	320091	106.72	214.23	2.01	2.04E-03	0.0648	anoctamin 4
Gca	227960	54.71	109.76	2.01	2.08E-05	0.00576	grancalcin

Table III. GENES REGULATED IN THE ABSENCE OF GATA4.

Genes are separated in downregulated and upregulated lists and organized by descending fold changes. The Symbol and EntrezID columns contain hyperlinks to the specific Gene page on the National Center for Biotechnology Information (NCBI) Entrez database.

APPENDIX A (continued)

Table IV: GENES DIFFERENTIALLY EXPRESSED AMONG WT AND GATA6^{GCKO}

34 genes were significant at p<0.01 with a fold change of 2 or more.

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA6^{gcko}				
Hist1h2ab	319172	122.56	30.1	-4.07	5.70E-03	0.45	histone cluster 1, H2ab
Gm3893	100042539	136.08	41.34	-3.29	1.39E-03	0.39	predicted gene 3893
4933409K07Rik	108816	147.97	45.67	-3.24	1.79E-03	0.39	RIKEN cDNA 4933409K07 gene
Krtap16-4	170654	86.41	30.4	-2.84	1.20E-04	0.32	keratin associated protein 16-4
Vmn2r43	381838	107.16	37.95	-2.82	4.83E-03	0.44	vomeronasal 2, receptor 43
Gm5891	545929	117.81	41.78	-2.82	6.00E-03	0.45	predicted gene 5891
Olfr1371	276865	103.25	38.2	-2.70	4.91E-03	0.44	olfactory receptor 1371
Rplp0	11837	662.73	246.13	-2.69	2.80E-06	0.08	ribosomal protein, large, P0
Comp	12845	491.63	184.43	-2.67	5.45E-05	0.3	cartilage oligomeric matrix protein
Vmn1r221	100312485	81.93	31	-2.64	2.80E-04	0.32	vomeronasal 1 receptor 221
Vmn1r79	100042437	96.91	36.8	-2.63	9.61E-04	0.37	vomeronasal 1 receptor 79

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA6^{gcko}				
Olfr1373	211472	93.44	35.54	-2.63	3.21E-03	0.43	olfactory receptor 1373
1700024J04Rik	71848	146.02	56.62	-2.58	9.96E-04	0.37	RIKEN cDNA 1700024J04 gene
C130026I21Rik	620078	92.39	36.99	-2.50	1.64E-03	0.39	RIKEN cDNA C130026I21 gene
A430089I19Rik	331195	95.6	39.61	-2.41	3.02E-03	0.43	RIKEN cDNA A430089I19 gene
Gm5458	432825	70.55	29.4	-2.40	2.24E-03	0.4	predicted gene 5458
Gm13271	435791	77.23	32.49	-2.38	1.02E-03	0.37	predicted gene 13271
Igk	243469	117.33	49.46	-2.37	7.03E-03	0.46	immunoglobulin kappa chain complex
Gm5168	382275	93.58	40.21	-2.33	9.83E-03	0.48	predicted gene 5168
Akr1c18	105349	124.21	54.86	-2.26	9.53E-04	0.37	aldo-keto reductase family 1, member C18
Grem2	23893	326.25	144.62	-2.26	5.58E-03	0.45	gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis)
Mpp7	75739	56.92	25.7	-2.21	4.93E-03	0.44	membrane protein, palmitoylated 7 (MAGUK p55 subfamily member 7)
Olfr1200	257887	63.67	29.32	-2.17	3.52E-04	0.33	olfactory receptor 1200

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA6^{gcko}				
Vmn2r60	637898	85.61	39.84	-2.15	7.48E-03	0.47	vomeronasal 2, receptor 60
Adh1	11522	488.29	227.88	-2.14	6.16E-03	0.45	alcohol dehydrogenase 1 (class I)
Acp1	11431	82.06	38.77	-2.12	2.72E-04	0.32	acid phosphatase 1, soluble
4933402N22Rik	545732	72.34	34.94	-2.07	4.69E-03	0.44	RIKEN cDNA 4933402N22 gene
Cma2	545055	57.06	27.69	-2.06	2.69E-04	0.32	chymase 2, mast cell
Pdgfrl	68797	60.45	29.52	-2.05	1.63E-03	0.39	platelet-derived growth factor receptor-like
Gm5114	330513	64.04	31.42	-2.04	3.81E-03	0.43	predicted gene 5114
Dad1	13135	651.42	320.2	-2.03	1.57E-03	0.39	defender against cell death 1
Gstm6	14867	120.79	59.45	-2.03	8.43E-03	0.47	glutathione S-transferase, mu 6
Olfr533	258056	63.15	31.17	-2.03	1.38E-03	0.39	olfactory receptor 533
Amy1	11722	68.46	33.88	-2.02	3.41E-04	0.33	amylase 1, salivary

Table IV. GENES REGULATED IN THE ABSENCE OF GATA6.

The Symbol and EntrezID columns contain hyperlinks to the specific Gene page on the NCBI Entrez database.

APPENDIX A (continued)

Table V: GENES DIFFERENTAILLY EXPRESSED AMONG WT AND GATA4/6^{GCKO}.

493 genes were significant at p<0.01 with a fold change of 2 or more

Downregulated Genes

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Comp	12845	491.63	25.2	-19.51	1.00E-07	1.21E-04	cartilage oligomeric matrix protein
Masp1	17174	549.15	48.09	-11.42	1.00E-07	1.00E-07	mannan-binding lectin serine peptidase 1
Cyp19a1	13075	1737.67	177.64	-9.78	1.81E-05	2.85E-03	cytochrome P450, family 19, subfamily a, polypeptide 1
Grem1	23892	332.4	49.98	-6.65	4.00E-07	2.63E-04	gremlin 1
Mro	71263	753.11	116.28	-6.48	1.00E-07	1.00E-07	maestro
Gabrb2	14401	194.09	30.04	-6.46	1.90E-06	6.47E-04	gamma-aminobutyric acid (GABA) A receptor, subunit beta 2
Pappa	18491	372.03	58.12	-6.40	3.90E-06	1.06E-03	pregnancy-associated plasma protein A
Plxnc1	54712	1571.83	303.27	-5.18	1.00E-07	1.21E-04	plexin C1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Fam162b	77296	139.99	28.18	-4.97	8.00E-07	3.73E-04	family with sequence similarity 162, member B
Inhba	16323	3079.31	647.09	-4.76	7.14E-04	2.80E-02	inhibin beta-A
Tnfsf11	21943	313.95	67.68	-4.64	1.00E-07	1.21E-04	tumor necrosis factor (ligand) superfamily, member 11
Tom1l1	71943	965.85	210.41	-4.59	6.00E-07	3.10E-04	target of myb1-like 1 (chicken)
Slc26a7	208890	1521.04	345.7	-4.40	1.53E-04	1.10E-02	solute carrier family 26, member 7
Enc1	13803	697.81	169.85	-4.11	3.22E-05	3.92E-03	ectodermal-neural cortex 1
Vmn2r43	381838	107.16	26.6	-4.03	7.53E-04	2.91E-02	vomeronasal 2, receptor 43
Pip5k1b	18719	166.39	41.89	-3.97	1.00E-07	1.21E-04	phosphatidylinositol-4-phosphate 5-kinase, type 1 beta
Gsta4	14860	659.42	171.93	-3.84	1.00E-07	1.00E-07	glutathione S-transferase, alpha 4
Alms1	236266	183.67	48.53	-3.78	1.00E-07	1.00E-07	Alstrom syndrome 1 homolog (human)
Rimklb	108653	682.67	180.82	-3.78	2.00E-07	1.56E-04	ribosomal modification protein rimK-like family member B

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Gabra1	14394	120.57	32.16	-3.75	2.00E-07	1.56E-04	gamma-aminobutyric acid (GABA) A receptor, subunit alpha 1
Plp1	18823	187.76	50.45	-3.72	4.95E-05	5.37E-03	proteolipid protein (myelin) 1
Slfn4	20558	105.56	29.2	-3.62	7.28E-03	1.09E-01	schlafen 4
Trib2	217410	949.48	262.98	-3.61	1.39E-04	1.04E-02	tribbles homolog 2 (Drosophila)
Lect1	16840	753.79	209.28	-3.60	1.10E-05	2.09E-03	leukocyte cell derived chemotaxin 1
Vcan	13003	1524.26	424.69	-3.59	8.70E-06	1.77E-03	versican
Dock5	68813	384.4	107.2	-3.59	5.20E-06	1.24E-03	dedicator of cytokinesis 5
Dock4	238130	628.53	179.85	-3.49	3.90E-06	1.06E-03	dedicator of cytokinesis 4
C130026I21Rik	620078	92.39	27.43	-3.37	2.85E-05	3.70E-03	RIKEN cDNA C130026I21 gene
Tox	252838	102	31.11	-3.28	1.00E-07	1.00E-07	thymocyte selection-associated high mobility group box
Ncrna00086	320237	186.54	56.94	-3.28	4.10E-06	1.06E-03	non-protein coding RNA 86
Satb2	212712	82.6	25.3	-3.26	8.52E-04	3.21E-02	special AT-rich sequence binding protein 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Cdh2	12558	440.14	134.92	-3.26	1.60E-06	5.94E-04	cadherin 2
D830030K20Rik	320333	84.14	26.1	-3.22	4.95E-04	2.23E-02	RIKEN cDNA D830030K20 gene
Ctsc	13032	183.52	58.35	-3.15	3.70E-06	1.05E-03	cathepsin C
Rhox8	434768	95.66	30.52	-3.13	2.40E-06	7.80E-04	reproductive homeobox 8
Nppc	18159	356.01	115.78	-3.07	6.36E-04	2.60E-02	natriuretic peptide type C
Gch1	14528	213.83	70.36	-3.04	1.73E-04	1.18E-02	GTP cyclohydrolase 1
Defb19	246700	233.75	76.95	-3.04	6.00E-07	3.10E-04	defensin beta 19
Mid1ip1	68041	626.43	207.57	-3.02	1.00E-07	1.00E-07	Mid1 interacting protein 1 (gastrulation specific G12-like (zebrafish))
Lypd6	320343	116.1	38.75	-3.00	5.88E-05	5.92E-03	LY6/PLAUR domain containing 6
Snord7	100302731	157.58	52.97	-2.97	1.56E-04	1.10E-02	small nucleolar RNA, C/D box 7
Fshr	14309	720.69	243.29	-2.96	3.00E-07	2.23E-04	follicle stimulating hormone receptor
Adamts1	11504	195.47	65.99	-2.96	1.00E-07	1.00E-07	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Inhbb	16324	1354.95	460.75	-2.94	3.39E-04	1.76E-02	inhibin beta-B
Pik3cg	30955	84.47	28.8	-2.93	5.55E-04	2.41E-02	phosphoinositide-3-kinase, catalytic, gamma polypeptide
Hey2	15214	738.55	252.79	-2.92	5.68E-05	5.79E-03	hairy/enhancer-of-split related with YRPW motif 2
Gpr126	215798	75.67	25.99	-2.91	7.00E-07	3.38E-04	G protein-coupled receptor 126
Tmeff2	56363	89.39	31.1	-2.87	1.01E-04	8.41E-03	transmembrane protein with EGF-like and two follistatin-like domains 2
Tulp2	56734	82.16	28.7	-2.86	7.22E-05	6.79E-03	tubby-like protein 2
Hcn1	15165	292.56	102.67	-2.85	2.45E-04	1.43E-02	hyperpolarization-activated, cyclic nucleotide-gated K+ 1
Adipor2	68465	682.33	240.32	-2.84	5.00E-07	2.89E-04	adiponectin receptor 2
Xlr5a	574438	72.09	25.5	-2.83	4.10E-04	1.97E-02	X-linked lymphocyte-regulated 5A
Fdx1	14148	325.95	115.66	-2.82	2.40E-03	5.89E-02	ferredoxin 1
Bub1	12235	395.33	140.91	-2.81	2.96E-04	1.61E-02	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Arrdc4	66412	117.14	41.84	-2.80	1.00E-07	1.21E-04	arrestin domain containing 4
Jak1	16451	1775.64	637.6	-2.78	1.14E-03	3.76E-02	Janus kinase 1
Aldh1a1	11668	1182.77	427.78	-2.76	1.43E-05	2.43E-03	aldehyde dehydrogenase family 1, subfamily A1
Coasy	71743	398.62	144.23	-2.76	8.80E-06	1.78E-03	Coenzyme A synthase
Ccdc68	381175	82.5	30.27	-2.73	4.13E-04	1.97E-02	coiled-coil domain containing 68
Atp10a	11982	569.48	210.76	-2.70	2.40E-06	7.80E-04	ATPase, class V, type 10A
Rassf2	215653	216.87	80.75	-2.69	3.87E-04	1.93E-02	Ras association (RalGDS/AF-6) domain family member 2
Gm129	229599	255.58	95.3	-2.68	1.95E-05	2.95E-03	predicted gene 129
Lhcgr	16867	2264.66	845.49	-2.68	5.64E-03	9.43E-02	luteinizing hormone/choriogonadotropin receptor
Rasgrp4	233046	107.07	40.02	-2.68	3.61E-05	4.28E-03	RAS guanyl releasing protein 4
Ralgapa2	241694	253.11	94.69	-2.67	5.00E-07	2.89E-04	Ral GTPase activating protein, alpha subunit 2 (catalytic)
Slc16a3	80879	194.39	73.29	-2.65	9.00E-07	4.13E-04	solute carrier family 16 (monocarboxylic acid transporters), member 3

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Idi1	319554	209.31	79.03	-2.65	8.60E-06	1.77E-03	isopentenyl-diphosphate delta isomerase
Lzts1	211134	161.35	62.05	-2.60	5.70E-06	1.31E-03	leucine zipper, putative tumor suppressor 1
Adh1	11522	488.29	187.96	-2.60	1.96E-03	5.25E-02	alcohol dehydrogenase 1 (class I)
Erdr1	170942	1645.66	636.27	-2.59	9.23E-03	1.25E-01	erythroid differentiation regulator 1
Chst5	56773	65.16	25.2	-2.59	6.33E-03	1.01E-01	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5
Slc12a7	20499	353.05	136.55	-2.59	2.17E-04	1.35E-02	solute carrier family 12, member 7
St3gal4	20443	718.79	279.82	-2.57	3.77E-04	1.89E-02	ST3 beta-galactoside alpha-2,3-sialyltransferase 4
Mir503	723879	98.36	39.38	-2.50	3.34E-03	7.11E-02	microRNA 503
Jam2	67374	957.69	383.54	-2.50	8.12E-05	7.48E-03	junction adhesion molecule 2
Gstm6	14867	120.79	48.57	-2.49	3.25E-03	7.00E-02	glutathione S-transferase, mu 6
Wapal	218914	363.48	146.34	-2.48	4.46E-04	2.09E-02	wings apart-like homolog (Drosophila)
Mapkbp1	26390	228.11	91.85	-2.48	4.05E-04	1.97E-02	mitogen-activated protein kinase binding protein 1
Srbd1	78586	271.03	109.75	-2.47	6.00E-07	3.10E-04	S1 RNA binding domain 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Tes	21753	192.47	78.52	-2.45	9.20E-06	1.85E-03	testis derived transcript
Prkar2b	19088	2328.29	950.06	-2.45	7.02E-04	2.77E-02	protein kinase, cAMP dependent regulatory, type II beta
Hipk2	15258	788.41	322.27	-2.45	3.00E-06	9.44E-04	homeodomain interacting protein kinase 2
Elovl2	54326	70.45	28.85	-2.44	7.00E-07	3.38E-04	elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2
Ces1b	382044	61.86	25.5	-2.43	8.63E-04	3.23E-02	carboxylesterase 1B
Gdnf	14573	208.44	86.27	-2.42	2.08E-05	3.10E-03	glial cell line derived neurotrophic factor
Rassf4	213391	103.62	43.04	-2.41	6.83E-04	2.74E-02	Ras association (RalGDS/AF-6) domain family member 4
Map3k5	26408	545.41	227.52	-2.40	2.28E-05	3.25E-03	mitogen-activated protein kinase kinase kinase 5
Klc2	16594	378.59	158.04	-2.40	3.00E-07	2.23E-04	kinesin light chain 2
Bex4	406217	243.93	102.05	-2.39	1.75E-04	1.18E-02	brain expressed gene 4
Acp1	11431	82.06	34.53	-2.38	3.84E-05	4.49E-03	acid phosphatase 1, soluble
Amy1	11722	68.46	28.86	-2.37	5.22E-04	2.32E-02	amylase 1, salivary

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Ext1	14042	1092.86	460.96	-2.37	1.98E-04	1.28E-02	exostoses (multiple) 1
Spin2	278240	192.93	81.51	-2.37	5.00E-07	2.89E-04	spindlin family, member 2
Calm1	12313	1102.45	466.4	-2.36	7.95E-05	7.40E-03	calmodulin 1
Abcd2	26874	167.06	70.91	-2.36	1.23E-04	9.60E-03	ATP-binding cassette, sub-family D (ALD), member 2
Sema3g	218877	165.87	70.84	-2.34	4.18E-05	4.74E-03	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G
Pla2g4a	18783	190.83	81.86	-2.33	1.20E-05	2.18E-03	phospholipase A2, group IVA (cytosolic, calcium-dependent)
Ccbl2	229905	157.85	67.9	-2.32	7.80E-06	1.62E-03	cysteine conjugate-beta lyase 2
BC057022	433940	408.18	176.56	-2.31	9.85E-04	3.45E-02	cDNA sequence BC057022
Hsd17b1	15485	2796.08	1214.57	-2.30	2.90E-04	1.59E-02	hydroxysteroid (17-beta) dehydrogenase 1
Piga	18700	61.94	26.91	-2.30	4.00E-07	2.63E-04	phosphatidylinositol glycan anchor biosynthesis, class A
Pdgfrl	68797	60.45	26.4	-2.29	2.99E-03	6.63E-02	platelet-derived growth factor receptor-like

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Mfsd7c	217721	93.19	40.76	-2.29	2.14E-05	3.13E-03	major facilitator superfamily domain containing 7C
Asah2	54447	413.66	181.69	-2.28	3.54E-05	4.25E-03	N-acylsphingosine amidohydrolase 2
Gm10406	100038847	63.61	28.07	-2.27	3.34E-04	1.75E-02	predicted gene 10406
Phex	18675	218.78	96.65	-2.26	2.92E-04	1.60E-02	phosphate regulating gene with homologies to endopeptidases on the X chromosome
Grem2	23893	326.25	144.17	-2.26	5.71E-03	9.48E-02	gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis)
Mapre2	212307	436.71	193.29	-2.26	5.39E-05	5.69E-03	microtubule-associated protein, RP/EB family, member 2
Pparg	19016	447.23	198.11	-2.26	3.12E-05	3.84E-03	peroxisome proliferator activated receptor gamma
Myo6	17920	373.01	165.44	-2.25	1.80E-06	6.20E-04	myosin VI
Hist1h2br	665622	133.81	59.35	-2.25	1.70E-06	6.07E-04	histone cluster 1 H2br
Prelid2	77619	87.68	38.94	-2.25	7.00E-07	3.38E-04	PRELI domain containing 2
Csrp2	13008	670.17	298.22	-2.25	3.46E-04	1.79E-02	cysteine and glycine-rich protein 2
Usp3	235441	819.53	364.69	-2.25	2.46E-04	1.43E-02	ubiquitin specific peptidase 3

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Speer8-ps1	74062	159.57	71.27	-2.24	5.15E-04	2.30E-02	spermatogenesis associated glutamate (E)-rich protein 8, pseudogene 1
Tmsb15l	399591	60.49	27.2	-2.22	3.47E-04	1.79E-02	thymosin beta 15b like
Gprc6a	210198	70.1	31.6	-2.22	4.59E-03	8.38E-02	G protein-coupled receptor, family C, group 6, member A
Epha7	13841	109.4	49.45	-2.21	1.14E-04	9.09E-03	Eph receptor A7
Fbxl22	74165	122.11	55.22	-2.21	3.25E-04	1.72E-02	F-box and leucine-rich repeat protein 22
Tmem45a	56277	335.16	151.79	-2.21	3.06E-03	6.73E-02	transmembrane protein 45a
Cdkn2c	12580	56.93	25.84	-2.20	1.50E-03	4.49E-02	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
Rps6ka2	20112	551.57	251.07	-2.20	2.20E-04	1.35E-02	ribosomal protein S6 kinase, polypeptide 2
Ddah1	69219	332.56	151.61	-2.19	1.30E-06	5.15E-04	dimethylarginine dimethylaminohydrolase 1
Slc38a5	209837	777.85	355.08	-2.19	1.20E-06	4.89E-04	solute carrier family 38, member 5
Ryr2	20191	65.56	29.95	-2.19	2.27E-05	3.25E-03	ryanodine receptor 2, cardiac
P2ry13	74191	58.01	26.52	-2.19	3.64E-04	1.86E-02	purinergic receptor P2Y, G-protein coupled 13

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Igsf3	78908	238.2	109.95	-2.17	4.10E-06	1.06E-03	immunoglobulin superfamily, member 3
Ephx2	13850	1024.09	473.45	-2.16	6.70E-04	2.70E-02	epoxide hydrolase 2, cytoplasmic
Ldlr	16835	603.79	279.28	-2.16	8.60E-05	7.63E-03	low density lipoprotein receptor
Etl4	208618	193.04	89.44	-2.16	3.70E-06	1.05E-03	enhancer trap locus 4
Fam13a	58909	1048.1	486.48	-2.15	5.25E-04	2.32E-02	family with sequence similarity 13, member A
Cnot6	104625	882.87	409.85	-2.15	1.00E-03	3.49E-02	CCR4-NOT transcription complex, subunit 6
Rnd2	11858	279.8	132.31	-2.11	3.05E-03	6.72E-02	Rho family GTPase 2
Ralb	64143	152.67	72.31	-2.11	2.66E-05	3.52E-03	v-ral simian leukemia viral oncogene homolog B (ras related)
Nckap5	210356	82.97	39.51	-2.10	9.26E-04	3.34E-02	NCK-associated protein 5
Hist1h2bb	319178	331.36	158.31	-2.09	1.14E-03	3.77E-02	histone cluster 1, H2bb
Hmgcr	15357	616.49	295.31	-2.09	2.04E-05	3.06E-03	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
Neb	17996	80.1	38.4	-2.09	2.49E-04	1.44E-02	nebulin
Mfsd2a	76574	383.55	184.58	-2.08	9.40E-06	1.88E-03	major facilitator superfamily domain containing 2A

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Rnf128	66889	87.81	42.3	-2.08	2.37E-03	5.86E-02	ring finger protein 128
Scgb3a1	68662	76.24	36.73	-2.08	5.10E-04	2.28E-02	secretoglobin, family 3A, member 1
Gcnt4	218476	84.68	40.81	-2.07	4.20E-06	1.07E-03	glucosaminyl (N-acetyl) transferase 4, core 2 (beta-1,6-N-acetylglucosaminyltransferase)
Chst1	76969	78.58	37.9	-2.07	2.65E-03	6.24E-02	carbohydrate (keratan sulfate Gal-6) sulfotransferase 1
Lrrc2	74249	375.61	182.23	-2.06	1.09E-03	3.65E-02	leucine rich repeat containing 2
Sdf2l1	64136	296.84	144.04	-2.06	2.70E-03	6.28E-02	stromal cell-derived factor 2-like 1
Slc7a8	50934	902.23	438.32	-2.06	3.04E-05	3.78E-03	solute carrier family 7 (cationic amino acid transporter, y+ system), member 8
Greb1l	381157	1057.89	517.79	-2.04	8.91E-04	3.27E-02	growth regulation by estrogen in breast cancer-like
Bnip3	12176	620.77	304.89	-2.04	6.87E-04	2.75E-02	BCL2/adenovirus E1B interacting protein 3
Cyb5	109672	2261.3	1113.23	-2.03	4.62E-04	2.15E-02	cytochrome b-5
Raver2	242570	132.79	65.45	-2.03	2.72E-05	3.58E-03	ribonucleoprotein, PTB-binding 2
Hmgb2	97165	191.93	94.82	-2.02	9.58E-03	1.28E-01	high mobility group box 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Dsel	319901	155.47	77.41	-2.01	2.35E-05	3.30E-03	dermatan sulfate epimerase-like
Zcchc16	619287	71.34	35.57	-2.01	6.03E-05	5.92E-03	zinc finger, CCHC domain containing 16
Zdhhc2	70546	185.1	92.36	-2.00	3.64E-05	4.28E-03	zinc finger, DHHC domain containing 2
Il1r1	16177	92.39	46.12	-2.00	4.68E-04	2.17E-02	interleukin 1 receptor, type I
Pgk1	18655	208.07	103.91	-2.00	1.14E-04	9.09E-03	phosphoglycerate kinase 1
Tnni3	21954	690.23	344.81	-2.00	1.33E-05	2.33E-03	troponin I, cardiac 3

Upregulated Genes

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Penk	18619	53.86	632.64	11.75	1.48E-04	1.09E-02	preproenkephalin
Igfbp4	16010	97.95	1117.05	11.40	5.67E-05	5.79E-03	insulin-like growth factor binding protein 4

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Col12a1	12816	32.75	328.92	10.04	6.08E-05	5.94E-03	collagen, type XII, alpha 1
Hsd3b6	15497	29.09	280.73	9.65	1.60E-04	1.11E-02	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6
Pcsk6	18553	57.16	544.35	9.52	1.50E-06	5.71E-04	proprotein convertase subtilisin/kexin type 6
Slc18a2	214084	200.64	1641.51	8.18	7.00E-07	3.38E-04	solute carrier family 18 (vesicular monoamine), member 2
Tns4	217169	30.21	243.76	8.07	2.30E-06	7.65E-04	tensin 4
Mapk10	26414	49.12	380.87	7.75	1.00E-07	1.00E-07	mitogen-activated protein kinase 10
Bpifb5	228802	33.99	259.5	7.63	1.00E-07	1.00E-07	BPI fold containing family B, member 5
Grik3	14807	39.43	287.7	7.30	1.00E-07	1.00E-07	glutamate receptor, ionotropic, kainate 3
Gm3579	100041932	80.89	556.21	6.88	1.01E-03	3.50E-02	predicted gene 3579
Gpx3	14778	274.38	1710.99	6.24	1.66E-03	4.75E-02	glutathione peroxidase 3
Plxdc1	72324	28.78	177.89	6.18	1.00E-07	1.21E-04	plexin domain containing 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
E330013P04Rik	107376	37.43	218.98	5.85	3.40E-06	1.02E-03	RIKEN cDNA E330013P04 gene
Kcnip3	56461	68.37	389.67	5.70	1.30E-06	5.15E-04	Kv channel interacting protein 3, calsenilin
Ogn	18295	88.02	472.66	5.37	1.64E-03	4.72E-02	osteoglycin
Ssbp2	66970	124.13	639.28	5.15	1.20E-06	4.89E-04	single-stranded DNA binding protein 2
Gria3	53623	34.45	176.3	5.12	1.42E-04	1.05E-02	glutamate receptor, ionotropic, AMPA3 (alpha 3)
Ren1	19701	57.38	288.85	5.03	4.00E-06	1.06E-03	renin 1 structural
Tgfbi	21810	87.58	438.42	5.01	2.52E-04	1.46E-02	transforming growth factor, beta induced
Adck3	67426	76.17	373.03	4.90	1.00E-07	1.00E-07	aarF domain containing kinase 3
Ptgis	19223	130.05	623.43	4.79	1.41E-05	2.43E-03	prostaglandin I2 (prostacyclin) synthase
Amh	11705	96.02	455.67	4.75	4.42E-04	2.08E-02	anti-Mullerian hormone
Srpx2	68792	33.57	158.27	4.71	2.59E-03	6.13E-02	sushi-repeat-containing protein, X-linked 2
Mmp2	17390	87.42	410.4	4.69	1.71E-03	4.83E-02	matrix metallopeptidase 2
Apoe	11816	204.43	934.87	4.57	1.35E-05	2.35E-03	apolipoprotein E

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Bcan	12032	31.37	142.39	4.54	5.92E-05	5.92E-03	brevican
Lgi3	213469	42.59	187.74	4.41	8.15E-05	7.49E-03	leucine-rich repeat LGI family, member 3
Spon1	233744	61.99	270.65	4.37	7.10E-05	6.76E-03	spondin 1, (f-spondin) extracellular matrix protein
Nos2	18126	51.1	223.1	4.37	1.20E-06	4.89E-04	nitric oxide synthase 2, inducible
Gm13691	668119	179.06	774.6	4.33	2.00E-07	1.56E-04	CWC22 spliceosome-associated protein homolog pseudogene
Igdcc4	56741	39.93	171.89	4.31	5.05E-05	5.43E-03	immunoglobulin superfamily, DCC subclass, member 4
Cyp1b1	13078	282.01	1213.07	4.30	2.00E-07	1.56E-04	cytochrome P450, family 1, subfamily b, polypeptide 1
Enpp6	320981	63.86	267.74	4.19	8.73E-05	7.68E-03	ectonucleotide pyrophosphatase/phosphodiesterase 6
Nrip2	60345	46.67	193.95	4.16	1.00E-07	1.21E-04	nuclear receptor interacting protein 2
Pdcd1	18566	33.87	140.51	4.15	1.70E-06	6.07E-04	programmed cell death 1
Tmem171	380863	179.74	739.59	4.11	6.00E-07	3.10E-04	transmembrane protein 171
Gm5294	384244	41.94	170.03	4.05	1.33E-03	4.17E-02	predicted gene 5294

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Coch	12810	70.81	285.64	4.03	1.05E-05	2.04E-03	coagulation factor C homolog (Limulus polyphemus)
Gmpr	66355	40.49	163.02	4.03	1.81E-04	1.20E-02	guanosine monophosphate reductase
Mmd2	75104	122.75	491.14	4.00	1.22E-05	2.19E-03	monocyte to macrophage differentiation-associated 2
Emx2	13797	48.69	194.94	4.00	1.80E-06	6.20E-04	empty spiracles homolog 2 (Drosophila)
Tgfbr3	21814	130.72	522.02	3.99	3.60E-06	1.04E-03	transforming growth factor, beta receptor III
Doc2b	13447	45.52	180.1	3.96	4.78E-05	5.27E-03	double C2, beta
Agt	11606	244.77	961.25	3.93	1.47E-03	4.41E-02	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
Htra3	78558	49.11	192.49	3.92	1.06E-04	8.70E-03	HtrA serine peptidase 3
Gdpd3	68616	28.87	111.93	3.88	4.19E-03	8.03E-02	glycerophosphodiester phosphodiesterase domain containing 3
Col8a1	12837	45.76	176.57	3.86	1.00E-07	1.00E-07	collagen, type VIII, alpha 1
Gatm	67092	54.59	209.42	3.84	2.22E-03	5.60E-02	glycine amidinotransferase (L-arginine:glycine amidinotransferase)
Gxylt2	232313	45.44	172.72	3.80	1.38E-04	1.04E-02	glucoside xylosyltransferase 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
P4ha3	320452	25.2	94.37	3.74	1.24E-03	3.95E-02	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III
Aspn	66695	37	137.54	3.72	4.64E-03	8.42E-02	asporin
S100b	20203	25.4	93.59	3.68	1.98E-03	5.29E-02	S100 protein, beta polypeptide, neural
Bcat1	12035	247.02	905.09	3.66	2.87E-05	3.70E-03	branched chain aminotransferase 1, cytosolic
Gadd45g	23882	46.89	170.87	3.64	1.08E-05	2.07E-03	growth arrest and DNA-damage-inducible 45 gamma
Igfbp5	16011	475.39	1727.62	3.63	2.41E-03	5.89E-02	insulin-like growth factor binding protein 5
Itih5	209378	113.38	409.02	3.61	2.09E-03	5.42E-02	inter-alpha (globulin) inhibitor H5
Spinlw1	75526	53.62	192.65	3.59	2.52E-03	6.04E-02	serine protease inhibitor-like, with Kunitz and WAP domains 1 (eppin)
Prkcb	18751	29.91	107.17	3.58	2.66E-05	3.52E-03	protein kinase C, beta
Fstl3	83554	47.59	169.31	3.56	5.31E-04	2.34E-02	follistatin-like 3
Mamdc2	71738	25.5	90.78	3.56	1.57E-04	1.10E-02	MAM domain containing 2
Pdgfra	18595	55.49	193.89	3.49	1.71E-03	4.84E-02	platelet derived growth factor receptor, alpha polypeptide

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Rgs9	19739	28.82	99.71	3.46	2.12E-05	3.13E-03	regulator of G-protein signaling 9
AB041803	232685	63.22	217.5	3.44	1.86E-05	2.86E-03	cDNA sequence AB041803
Ptprz1	19283	38.95	133.86	3.44	3.00E-05	3.77E-03	protein tyrosine phosphatase, receptor type Z, polypeptide 1
Fbln7	70370	35.09	120.71	3.44	1.31E-04	1.00E-02	fibulin 7
Ace	11421	34.36	118.26	3.44	1.58E-04	1.10E-02	angiotensin I converting enzyme (peptidyl-dipeptidase A) 1
Fbln2	14115	55.82	187.82	3.36	1.61E-04	1.12E-02	fibulin 2
Slc45a4	106068	181.68	597.2	3.29	1.20E-06	4.89E-04	solute carrier family 45, member 4
Kcnma1	16531	94.72	310.94	3.28	3.50E-06	1.02E-03	potassium large conductance calcium-activated channel, subfamily M, alpha member 1
Sorl1	20660	61.22	201.01	3.28	2.49E-05	3.41E-03	sortilin-related receptor, LDLR class A repeats-containing
Adhfe1	76187	51.95	170.2	3.28	4.00E-07	2.63E-04	alcohol dehydrogenase, iron containing, 1
Lyz2	17105	103.85	338.14	3.26	8.74E-03	1.21E-01	lysozyme 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Slc10a4	231290	57.72	186.39	3.23	3.80E-06	1.06E-03	solute carrier family 10 (sodium/bile acid cotransporter family), member 4
Lamc3	23928	52.66	170	3.23	2.07E-04	1.32E-02	laminin gamma 3
Spp1	20750	48.39	156.37	3.23	9.49E-04	3.39E-02	secreted phosphoprotein 1
Tnfrsf21	94185	55.94	179.43	3.21	1.36E-04	1.03E-02	tumor necrosis factor receptor superfamily, member 21
Kcnt1	227632	81.81	261.9	3.20	5.00E-07	2.89E-04	potassium channel, subfamily T, member 1
Rarres2	71660	69.32	222.07	3.20	4.86E-04	2.22E-02	retinoic acid receptor responder (tazarotene induced) 2
Kcnk1	16525	75.98	239.72	3.15	2.30E-04	1.37E-02	potassium channel, subfamily K, member 1
Phyhd1	227696	33.85	105.54	3.12	9.57E-05	8.12E-03	phytanoyl-CoA dioxygenase domain containing 1
Fam198a	245050	27.48	85.46	3.11	1.10E-04	8.94E-03	family with sequence similarity 198, member A
Ptgds	19215	72.89	224.88	3.09	8.72E-05	7.68E-03	prostaglandin D2 synthase (brain)
Pcp4l1	66425	78.18	240.77	3.08	3.02E-05	3.78E-03	Purkinje cell protein 4-like 1
Lama2	16773	50.67	155.8	3.07	1.20E-03	3.87E-02	laminin, alpha 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Atp2b4	381290	50.82	155.78	3.07	5.35E-04	2.35E-02	ATPase, Ca++ transporting, plasma membrane 4
Fam110c	104943	30.55	93.59	3.06	5.30E-06	1.25E-03	family with sequence similarity 110, member C
Gstt1	14871	40.59	123.37	3.04	7.80E-05	7.28E-03	glutathione S-transferase, theta 1
Gna14	14675	36.81	111.25	3.02	1.00E-07	1.21E-04	guanine nucleotide binding protein, alpha 14
Gdpd2	71584	60.55	181.66	3.00	2.12E-05	3.13E-03	glycerophosphodiester phosphodiesterase domain containing 2
Abcc3	76408	45.31	135.8	3.00	4.00E-07	2.63E-04	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
Hpgd	15446	315.89	940.27	2.98	3.50E-06	1.02E-03	hydroxyprostaglandin dehydrogenase 15 (NAD)
Cacna1h	58226	74.22	219.48	2.96	2.14E-05	3.13E-03	calcium channel, voltage-dependent, T type, alpha 1H subunit
Mgp	17313	145.41	428.26	2.95	4.42E-03	8.25E-02	matrix Gla protein
Usp18	24110	26.19	77.14	2.95	5.91E-05	5.92E-03	ubiquitin specific peptidase 18
Tspan4	64540	167.84	493.34	2.94	5.48E-03	9.28E-02	tetraspanin 4
Nipal1	70701	76.83	224.07	2.92	5.98E-05	5.92E-03	NIPA-like domain containing 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Ptch1	19206	213.69	618.93	2.90	5.37E-05	5.69E-03	patched homolog 1
Steap2	74051	78.99	228.49	2.89	9.97E-05	8.38E-03	six transmembrane epithelial antigen of prostate 2
Tmem35	67564	54.79	158.56	2.89	1.60E-04	1.11E-02	transmembrane protein 35
Trpc4	22066	27.09	78.04	2.88	2.77E-03	6.36E-02	transient receptor potential cation channel, subfamily C, member 4
Slc38a3	76257	284.48	815.52	2.87	1.51E-04	1.10E-02	solute carrier family 38, member 3
Rorc	19885	38.62	110.94	2.87	7.40E-06	1.59E-03	RAR-related orphan receptor gamma
Hmga2	15364	42.09	120.38	2.86	4.20E-06	1.07E-03	high mobility group AT-hook 2
Klhl31	244923	69.71	198.44	2.85	2.80E-05	3.65E-03	kelch-like 31 (Drosophila)
Pvt1	19296	35.53	101.18	2.85	4.22E-05	4.75E-03	plasmacytoma variant translocation 1
Gadd45b	17873	51.21	144.9	2.83	3.62E-05	4.28E-03	growth arrest and DNA-damage-inducible 45 beta
Drp2	13497	29.65	84	2.83	1.80E-06	6.20E-04	dystrophin related protein 2
Gadd45a	13197	56.91	160.58	2.82	2.21E-05	3.21E-03	growth arrest and DNA-damage-inducible 45 alpha

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Camsap3	69697	39.79	112.26	2.82	4.05E-04	1.97E-02	calmodulin regulated spectrin-associated protein family, member 3
Zp3	22788	179.09	502.49	2.81	2.01E-03	5.30E-02	zona pellucida glycoprotein 3
Zp2	22787	98.41	275.27	2.80	1.50E-03	4.49E-02	zona pellucida glycoprotein 2
Leprel1	210530	69.58	194.62	2.80	6.19E-04	2.56E-02	leprecan-like 1
Tmeff1	230157	43.98	123.11	2.80	1.60E-06	5.94E-04	transmembrane protein with EGF-like and two follistatin-like domains 1
Itih2	16425	33.22	92.62	2.79	4.50E-06	1.13E-03	inter-alpha trypsin inhibitor, heavy chain 2
Mblac2	72852	97.31	270.03	2.78	5.40E-06	1.26E-03	metallo-beta-lactamase domain containing 2
Apcdd1	494504	79.07	219.9	2.78	6.70E-06	1.46E-03	adenomatosis polyposis coli down-regulated 1
Limch1	77569	40.35	112.33	2.78	5.10E-06	1.24E-03	LIM and calponin homology domains 1
Klf9	16601	88.86	245.9	2.77	6.00E-07	3.10E-04	Kruppel-like factor 9
Nrp2	18187	40.83	112.37	2.75	1.68E-03	4.76E-02	neuropilin 2
Dkkl1	50722	28.04	77.16	2.75	2.19E-04	1.35E-02	dickkopf-like 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Cxx1c	72865	92.61	252.4	2.73	8.95E-05	7.80E-03	CAAX box 1 homolog C (human)
Nupr1	56312	90.12	246.34	2.73	5.00E-07	2.89E-04	nuclear protein 1
Oplah	75475	76.45	208.61	2.73	4.45E-04	2.09E-02	5-oxoprolinase (ATP-hydrolysing)
Gdpd5	233552	68.55	187.47	2.73	3.39E-03	7.17E-02	glycerophosphodiester phosphodiesterase domain containing 5
Ctsf	56464	71.87	195.75	2.72	2.89E-04	1.59E-02	cathepsin F
Itgb8	320910	45.67	124.19	2.72	1.31E-05	2.31E-03	integrin beta 8
B4galt1	14595	273.64	740.11	2.70	4.10E-06	1.06E-03	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1
S100a1	20193	182.34	491.43	2.70	3.98E-03	7.77E-02	S100 calcium binding protein A1
Sned1	208777	98.63	266.17	2.70	1.20E-06	4.89E-04	sushi, nidogen and EGF-like domains 1
Bcl2l10	12049	80.14	215.47	2.69	6.65E-03	1.04E-01	Bcl2-like 10
Itm2a	16431	79.76	214.46	2.69	5.57E-03	9.34E-02	integral membrane protein 2A
Uchl1	22223	137.19	367.86	2.68	5.34E-03	9.16E-02	ubiquitin carboxy-terminal hydrolase L1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Wisp2	22403	94.42	252.58	2.68	7.17E-05	6.79E-03	WNT1 inducible signaling pathway protein 2
Sox18	20672	85.85	230.05	2.68	4.19E-05	4.74E-03	SRY-box containing gene 18
Pltp	18830	41.33	110.47	2.67	1.23E-03	3.95E-02	phospholipid transfer protein
Ccno	218630	61.99	163.73	2.64	1.23E-03	3.95E-02	cyclin O
Arhgap42	71544	301.17	790.77	2.63	6.00E-06	1.36E-03	Rho GTPase activating protein 42
Map1lc3a	66734	122.65	323.15	2.63	5.24E-04	2.32E-02	microtubule-associated protein 1 light chain 3 alpha
Rnf19b	75234	97.87	257.74	2.63	6.00E-07	3.10E-04	ring finger protein 19B
Mosc2	67247	72.74	189.12	2.60	7.63E-04	2.94E-02	MOCO sulphurase C-terminal domain containing 2
Ror1	26563	60.59	157.24	2.60	7.50E-06	1.60E-03	receptor tyrosine kinase-like orphan receptor 1
Wfdc10	629756	135.01	349.78	2.59	1.57E-04	1.10E-02	WAP four-disulfide core domain 10
Zp1	22786	84.75	219.19	2.59	3.53E-03	7.33E-02	zona pellucida glycoprotein 1
Angptl1	72713	72.66	187.97	2.59	2.44E-05	3.36E-03	angiopoietin-like 1
Adarb1	110532	36.61	94.88	2.59	2.00E-07	1.56E-04	adenosine deaminase, RNA-specific, B1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Bcl6	12053	32.45	84.01	2.59	1.81E-04	1.20E-02	B cell leukemia/lymphoma 6
Gem	14579	49.33	127.04	2.58	3.64E-05	4.28E-03	GTP binding protein (gene overexpressed in skeletal muscle)
Selenbp1	20341	35.14	90.61	2.58	5.85E-04	2.48E-02	selenium binding protein 1
Ggt5	23887	32.41	83.57	2.58	1.25E-05	2.23E-03	gamma-glutamyltransferase 5
Adamts2	216725	265.13	681.43	2.57	7.60E-06	1.61E-03	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 2
Gas6	14456	330.72	846.87	2.56	3.58E-03	7.37E-02	growth arrest specific 6
Axl	26362	270.76	694.35	2.56	1.97E-04	1.28E-02	AXL receptor tyrosine kinase
Cwc22	80744	49.17	126.01	2.56	3.22E-05	3.92E-03	CWC22 spliceosome-associated protein homolog (S. cerevisiae)
Gpr165	76206	105.28	268.2	2.55	3.50E-06	1.02E-03	G protein-coupled receptor 165
Padi6	242726	94.94	241.75	2.55	6.87E-03	1.06E-01	peptidyl arginine deiminase, type VI
Dbp	13170	93.82	237.34	2.53	5.81E-04	2.48E-02	D site albumin promoter binding protein

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Abcc4	239273	56.16	142.27	2.53	2.34E-05	3.30E-03	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
Fbp1	14121	30.31	76.8	2.53	3.00E-06	9.44E-04	fructose bisphosphatase 1
Fabp3	14077	89.53	225.91	2.52	2.06E-03	5.35E-02	fatty acid binding protein 3, muscle and heart
Mxra8	74761	121.23	304.57	2.51	4.03E-03	7.80E-02	matrix-remodelling associated 8
Sgk3	170755	57.19	143.74	2.51	1.18E-05	2.16E-03	serum/glucocorticoid regulated kinase 3
Kcnk5	16529	37.78	95.02	2.51	8.97E-04	3.28E-02	potassium channel, subfamily K, member 5
Rasgrp1	19419	38.85	96.92	2.49	2.25E-05	3.24E-03	RAS guanyl releasing protein 1
Hpse	15442	28.68	71.3	2.49	6.15E-04	2.55E-02	heparanase
Deptor	97998	81.99	203.62	2.48	2.50E-05	3.41E-03	DEP domain containing MTOR-interacting protein
Mrc2	17534	39.9	98.85	2.48	1.74E-03	4.89E-02	mannose receptor, C type 2
Cldn11	18417	39.84	98.83	2.48	6.19E-03	9.96E-02	claudin 11
Mkx	210719	153.61	378.9	2.47	2.57E-05	3.45E-03	mohawk homeobox
Slc25a42	73095	103.33	254.87	2.47	9.11E-04	3.30E-02	solute carrier family 25, member 42

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Cfhr2	545366	92.55	228.54	2.47	4.44E-03	8.26E-02	complement factor H-related 2
Maml2	270118	68.07	167.98	2.47	1.10E-06	4.89E-04	mastermind like 2 (Drosophila)
Sh3pxd2a	14218	38.17	93.76	2.46	2.55E-04	1.47E-02	SH3 and PX domains 2A
Eya4	14051	33.94	83.04	2.45	8.55E-05	7.61E-03	eyes absent 4 homolog (Drosophila)
H2-D1	14964	178	433.32	2.43	6.92E-04	2.76E-02	histocompatibility 2, D region locus 1
Tcl1b1	27379	80.13	194.65	2.43	4.31E-03	8.14E-02	T cell leukemia/lymphoma 1B, 1
Slc43a2	215113	34.72	84.34	2.43	9.60E-06	1.89E-03	solute carrier family 43, member 2
Cpxm1	56264	69.47	167.45	2.41	2.08E-04	1.32E-02	carboxypeptidase X 1 (M14 family)
Gpnmb	93695	32.63	78.64	2.41	3.94E-05	4.54E-03	glycoprotein (transmembrane) nmb
Polr3g	67486	63.63	152.73	2.40	2.57E-05	3.45E-03	polymerase (RNA) III (DNA directed) polypeptide G
Slc7a4	224022	77.58	185.46	2.39	7.98E-04	3.04E-02	solute carrier family 7 (cationic amino acid transporter, y+ system), member 4
Sgpp2	433323	57.34	137.32	2.39	1.76E-05	2.80E-03	sphingosine-1-phosphate phosphotase 2
Ank2	109676	37.09	88.59	2.39	4.00E-07	2.63E-04	ankyrin 2, brain

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Dmrta1	242523	28.4	67.76	2.39	7.16E-04	2.81E-02	doublesex and mab-3 related transcription factor like family A1
Lamb1	16777	118.21	281.82	2.38	9.31E-05	8.02E-03	laminin B1
Akr1b10	67861	71.8	170.92	2.38	1.11E-05	2.10E-03	aldo-keto reductase family 1, member B10 (aldose reductase)
E330017A01Rik	224247	54.53	129.87	2.38	1.87E-03	5.10E-02	RIKEN cDNA E330017A01 gene
Slc19a3	80721	38.25	90.8	2.37	4.36E-04	2.06E-02	solute carrier family 19, member 3
Fcgrt	14132	198.18	468.44	2.36	1.97E-03	5.27E-02	Fc receptor, IgG, alpha chain transporter
Nbl1	17965	61.85	145.95	2.36	2.41E-05	3.36E-03	neuroblastoma, suppression of tumorigenicity 1
Sdk1	330222	38.87	91.63	2.36	2.00E-07	1.56E-04	sidekick homolog 1 (chicken)
Fgf11	14166	29.8	70.47	2.36	1.97E-03	5.27E-02	fibroblast growth factor 11
Clec2d	93694	67.78	158.99	2.35	4.22E-03	8.06E-02	C-type lectin domain family 2, member d
Dpysl5	65254	111.35	260.68	2.34	2.59E-05	3.45E-03	dihydropyrimidinase-like 5
A4galt	239559	62.43	146.29	2.34	5.02E-05	5.42E-03	alpha 1,4-galactosyltransferase

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Crabp2	12904	182.89	426.92	2.33	9.95E-04	3.47E-02	cellular retinoic acid binding protein II
Tbc1d9	71310	28.24	65.75	2.33	2.00E-07	1.56E-04	TBC1 domain family, member 9
Abhd14a	68644	74.18	171.5	2.31	4.44E-03	8.26E-02	abhydrolase domain containing 14A
Sorbs2	234214	64.2	148.6	2.31	1.04E-03	3.56E-02	sorbin and SH3 domain containing 2
Tmem176a	66058	154.39	355.41	2.30	6.87E-03	1.06E-01	transmembrane protein 176A
Chrd	12667	27.6	63.47	2.30	1.52E-03	4.50E-02	chordin
Itgb5	16419	239.5	548.33	2.29	2.87E-04	1.59E-02	integrin beta 5
Rgs11	50782	61.56	140.87	2.29	3.74E-03	7.58E-02	regulator of G-protein signaling 11
Slc24a3	94249	27.52	62.93	2.29	7.47E-03	1.10E-01	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3
Abca1	11303	158.66	361.84	2.28	9.77E-04	3.44E-02	ATP-binding cassette, sub-family A (ABC1), member 1
Lbp	16803	48.81	110.77	2.27	5.18E-03	9.00E-02	lipopolysaccharide binding protein
Derl3	70377	333.5	753.4	2.26	5.08E-04	2.28E-02	Der1-like domain family, member 3

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Nobox	18291	31.13	70.3	2.26	3.45E-03	7.25E-02	NOBOX oogenesis homeobox
Smoc1	64075	238.23	536.93	2.25	4.89E-05	5.36E-03	SPARC related modular calcium binding 1
Tfpi	21788	106.41	239.17	2.25	3.14E-04	1.69E-02	tissue factor pathway inhibitor
Olfml2b	320078	79.41	178.62	2.25	9.21E-03	1.25E-01	olfactomedin-like 2B
Ltbp3	16998	292.5	654.66	2.24	9.01E-05	7.82E-03	latent transforming growth factor beta binding protein 3
Ednra	13617	232.15	519.81	2.24	7.20E-06	1.56E-03	endothelin receptor type A
C87977	97187	164.69	368.49	2.24	3.07E-03	6.73E-02	expressed sequence C87977
Fam78a	241303	129.38	289.72	2.24	6.02E-05	5.92E-03	family with sequence similarity 78, member A
Nup210	54563	91.29	204.55	2.24	2.42E-05	3.36E-03	nucleoporin 210
Pknox2	208076	83.33	187.05	2.24	1.24E-04	9.64E-03	Pbx/knotted 1 homeobox 2
Tns1	21961	75.37	168.47	2.24	3.50E-04	1.80E-02	tensin 1
Sytl2	83671	39.54	88.66	2.24	5.00E-07	2.89E-04	synaptotagmin-like 2
Omd	27047	77.08	171.96	2.23	1.05E-04	8.69E-03	osteomodulin

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Plat	18791	70	155.99	2.23	1.05E-03	3.57E-02	plasminogen activator, tissue
B4galt6	56386	63.41	141.45	2.23	7.32E-03	1.09E-01	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6
Ptpre	19267	62.45	139.39	2.23	1.57E-05	2.60E-03	protein tyrosine phosphatase, receptor type, E
Plekhb2	226971	45.19	100.92	2.23	6.96E-04	2.77E-02	pleckstrin homology domain containing, family B (evectins) member 2
Ehd2	259300	131.76	292.39	2.22	2.64E-04	1.51E-02	EH-domain containing 2
Cited1	12705	113.36	251.11	2.22	3.48E-03	7.28E-02	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1
Tuba8	53857	98.93	219.57	2.22	2.22E-05	3.21E-03	tubulin, alpha 8
Cd200	17470	75.04	166.94	2.22	5.94E-03	9.73E-02	CD200 antigen
Fbln5	23876	46.07	102.11	2.22	8.09E-03	1.16E-01	fibulin 5
Lgals3bp	19039	93.44	206.25	2.21	3.86E-03	7.69E-02	lectin, galactoside-binding, soluble, 3 binding protein
Tmem231	234740	76.38	168.6	2.21	3.25E-03	7.00E-02	transmembrane protein 231
Cmbl	69574	50.45	111.03	2.20	2.22E-04	1.36E-02	carboxymethylenebutenolidase-like (Pseudomonas)

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Isyna1	71780	475.37	1039.12	2.19	6.03E-04	2.52E-02	myo-inositol 1-phosphate synthase A1
Myc	17869	191.44	419.24	2.19	6.36E-04	2.60E-02	myelocytomatosis oncogene
Smpdl3a	57319	125.28	274.88	2.19	1.61E-05	2.63E-03	sphingomyelin phosphodiesterase, acid-like 3A
Gpm6b	14758	116.64	255.16	2.19	7.80E-06	1.62E-03	glycoprotein m6b
Atp1b1	11931	209.85	458.2	2.18	6.30E-05	6.14E-03	ATPase, Na+/K+ transporting, beta 1 polypeptide
Rab11fip5	52055	67.46	146.8	2.18	3.13E-05	3.84E-03	RAB11 family interacting protein 5 (class I)
Gpr98	110789	55.99	121.91	2.18	1.10E-06	4.89E-04	G protein-coupled receptor 98
Lims2	225341	42.24	91.89	2.18	1.40E-06	5.40E-04	LIM and senescent cell antigen like domains 2
Spint2	20733	106.44	231.3	2.17	1.60E-03	4.65E-02	serine protease inhibitor, Kunitz type 2
H6pd	100198	98.12	212.54	2.17	2.98E-04	1.61E-02	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
Cpa1	109697	85.51	185.42	2.17	2.46E-03	5.95E-02	carboxypeptidase A1, pancreatic
Got2	14719	73	158.39	2.17	5.94E-03	9.73E-02	glutamate oxaloacetate transaminase 2, mitochondrial
Rfpl4	192658	63.83	138.18	2.16	4.30E-03	8.12E-02	ret finger protein-like 4

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Gyltl1b	228366	221.86	477.7	2.15	1.45E-05	2.45E-03	glycosyltransferase-like 1B
Tk2	57813	78.09	168.23	2.15	3.87E-03	7.70E-02	thymidine kinase 2, mitochondrial
Slc7a5	20539	676.74	1448.21	2.14	2.25E-04	1.36E-02	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
BC031353	235493	163.17	348.85	2.14	4.10E-06	1.06E-03	cDNA sequence BC031353
Rab34	19376	101.62	217.09	2.14	2.92E-03	6.51E-02	RAB34, member of RAS oncogene family
Tppp3	67971	73.19	156.3	2.14	8.69E-04	3.23E-02	tubulin polymerization-promoting protein family member 3
Cyp4f18	72054	50.64	108.43	2.14	7.95E-03	1.15E-01	cytochrome P450, family 4, subfamily f, polypeptide 18
Cdh3	12560	41.78	89.26	2.14	3.27E-03	7.02E-02	cadherin 3
Kcnh1	16510	38.96	83.31	2.14	1.40E-04	1.04E-02	potassium voltage-gated channel, subfamily H (eag-related), member 1
Slc4a11	269356	34.66	74.05	2.14	5.99E-05	5.92E-03	solute carrier family 4, sodium bicarbonate transporter-like, member 11
Ptges	64292	617.1	1314.54	2.13	5.65E-05	5.79E-03	prostaglandin E synthase

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Wt1	22431	79.08	168.13	2.13	3.93E-03	7.77E-02	Wilms tumor 1 homolog
Plin2	11520	69.21	147.7	2.13	6.70E-05	6.49E-03	perilipin 2
Rtn4rl1	237847	56.52	120.38	2.13	3.67E-03	7.46E-02	reticulon 4 receptor-like 1
Capg	12332	48.88	104.06	2.13	1.20E-03	3.87E-02	capping protein (actin filament), gelsolin-like
Sema4d	20354	45.45	97.01	2.13	4.00E-06	1.06E-03	semaphori 4D
Sema5b	20357	37.12	79.2	2.13	6.50E-06	1.43E-03	semaphorin 5B
Gpr20	239530	34.23	72.96	2.13	4.01E-04	1.96E-02	G protein-coupled receptor 20
Glul	14645	269.32	570.71	2.12	9.47E-03	1.27E-01	glutamate-ammonia ligase (glutamine synthetase)
Gm97	225923	140.47	298.3	2.12	1.75E-03	4.90E-02	predicted gene 97
Gdf9	14566	108.7	230.82	2.12	2.85E-03	6.44E-02	growth differentiation factor 9
Slit3	20564	62.85	133	2.12	3.50E-03	7.29E-02	slit homolog 3 (Drosophila)
Kdelc2	68304	61.92	131.15	2.12	2.30E-03	5.75E-02	KDEL (Lys-Asp-Glu-Leu) containing 2
Daam2	76441	50.77	107.67	2.12	1.61E-05	2.63E-03	dishevelled associated activator of morphogenesis 2

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Foxp2	114142	213.82	451.57	2.11	2.90E-06	9.33E-04	forkhead box P2
Entpd1	12495	201.82	425.12	2.11	1.18E-03	3.84E-02	ectonucleoside triphosphate diphosphohydrolase 1
Slc25a23	66972	154.14	324.74	2.11	1.31E-05	2.31E-03	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23
Eya2	14049	113.08	239.04	2.11	5.46E-05	5.75E-03	eyes absent 2 homolog (Drosophila)
Rbl2	19651	70.46	148.31	2.11	8.00E-05	7.42E-03	retinoblastoma-like 2
Car12	76459	58.02	122.7	2.11	6.53E-04	2.64E-02	carbonic anyhydrase 12
Lphn2	99633	268.16	562.99	2.10	1.02E-04	8.52E-03	latrophilin 2
Oog1	193322	167.72	351.52	2.10	4.41E-03	8.25E-02	oogenesin 1
Acot2	171210	126.94	267.03	2.10	1.79E-04	1.19E-02	acyl-CoA thioesterase 2
Setd7	73251	92.04	193.19	2.10	6.89E-05	6.60E-03	SET domain containing (lysine methyltransferase) 7
Tmem41a	66664	64.25	135.03	2.10	2.02E-03	5.30E-02	transmembrane protein 41a
Arg2	11847	35.78	75.1	2.10	2.20E-03	5.58E-02	arginase type II
Crip3	114570	31.33	65.72	2.10	8.06E-05	7.45E-03	cysteine-rich protein 3

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Sptlc2	20773	273.17	571	2.09	8.89E-04	3.27E-02	serine palmitoyltransferase, long chain base subunit 2
Cfh	12628	228.96	479.24	2.09	1.42E-03	4.31E-02	complement component factor h
Col6a1	12833	169.24	353.13	2.09	1.52E-04	1.10E-02	collagen, type VI, alpha 1
Angptl4	57875	115.66	241.78	2.09	1.33E-03	4.16E-02	angiopoietin-like 4
Qtrt1	60507	93.64	195.52	2.09	2.98E-03	6.60E-02	queuine tRNA-ribosyltransferase 1
Slc19a2	116914	82.68	172.44	2.09	1.22E-03	3.93E-02	solute carrier family 19 (thiamine transporter), member 2
C87414	381654	47.03	98.15	2.09	1.01E-03	3.50E-02	expressed sequence C87414
Papln	170721	46.23	96.67	2.09	3.55E-04	1.82E-02	papilin, proteoglycan-like sulfated glycoprotein
Nlrc5	434341	40.2	83.83	2.09	5.64E-04	2.43E-02	NLR family, CARD domain containing 5
Sema3d	108151	38.37	80	2.09	8.36E-03	1.18E-01	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D
Pipox	19193	34.28	71.73	2.09	3.90E-06	1.06E-03	pipecolic acid oxidase
Tmie	20776	29.53	61.84	2.09	9.60E-06	1.89E-03	transmembrane inner ear

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Hist1h2aa	319163	216.3	450.15	2.08	5.04E-03	8.86E-02	histone cluster 1, H2aa
Fbxw14	50757	136.51	283.53	2.08	7.09E-03	1.07E-01	F-box and WD-40 domain protein 14
Kctd14	233529	64.74	134.75	2.08	9.17E-03	1.25E-01	potassium channel tetramerisation domain containing 14
Zfp36l1	12192	57.05	118.59	2.08	3.99E-03	7.78E-02	zinc finger protein 36, C3H type-like 1
Sesn2	230784	53.75	111.74	2.08	1.31E-03	4.13E-02	sestrin 2
Hes1	15205	39.53	82.26	2.08	9.24E-04	3.34E-02	hairy and enhancer of split 1 (Drosophila)
Stc2	20856	38.27	79.57	2.08	9.97E-03	1.30E-01	stanniocalcin 2
Fmo1	14261	29.71	61.87	2.08	3.86E-05	4.49E-03	flavin containing monooxygenase 1
Igfbp2	16008	85.72	177.17	2.07	3.91E-03	7.75E-02	insulin-like growth factor binding protein 2
Acsf2	264895	77.98	161.63	2.07	6.10E-03	9.86E-02	acyl-CoA synthetase family member 2
Mxra7	67622	69.46	143.98	2.07	1.96E-04	1.28E-02	matrix-remodelling associated 7
Epha4	13838	64.26	133.27	2.07	9.95E-04	3.47E-02	Eph receptor A4
Gas1	14451	50.81	105.42	2.07	2.03E-03	5.32E-02	growth arrest specific 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Arhgdig	14570	38.93	80.59	2.07	3.86E-05	4.49E-03	Rho GDP dissociation inhibitor (GDI) gamma
Sik1	17691	111.56	229.35	2.06	1.65E-04	1.14E-02	salt inducible kinase 1
Flrt1	396184	51.4	106.03	2.06	3.10E-03	6.78E-02	fibronectin leucine rich transmembrane protein 1
Gm15698	217066	43.52	89.83	2.06	4.07E-03	7.87E-02	transcription elongation factor B (SIII), polypeptide 2 pseudogene
Phxr4	18689	33.9	69.89	2.06	1.43E-04	1.05E-02	per-hexamer repeat gene 4
H1foo	171506	58.66	119.98	2.05	1.73E-03	4.87E-02	H1 histone family, member O, oocyte-specific
Mansc1	67729	43.54	89.31	2.05	1.61E-03	4.68E-02	MANSC domain containing 1
B130016D09Rik	436015	27.62	56.55	2.05	6.20E-06	1.39E-03	RIKEN cDNA B130016D09 cDNA
Mmp23	26561	132.02	268.88	2.04	1.48E-03	4.43E-02	matrix metallopeptidase 23
Pdpn	14726	117.83	240.46	2.04	5.16E-03	8.98E-02	podoplanin
Fam126b	213056	106.26	216.92	2.04	1.31E-04	1.00E-02	family with sequence similarity 126, member B
Grasp	56149	103.81	212.14	2.04	7.39E-04	2.88E-02	GRP1 (general receptor for phosphoinositides 1)-associated scaffold protein

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Gpcpd1	74182	73.96	151.03	2.04	3.82E-03	7.67E-02	glycerophosphocholine phosphodiesterase GDE1 homolog (S. cerevisiae)
Msi1	17690	62.69	128.2	2.04	4.13E-04	1.97E-02	Musashi homolog 1(Drosophila)
Csdc2	105859	51.47	104.89	2.04	2.06E-03	5.35E-02	cold shock domain containing C2, RNA binding
Uggt2	66435	50.84	103.49	2.04	1.94E-03	5.22E-02	UDP-glucose glycoprotein glucosyltransferase 2
Cnp	12799	41.76	85.27	2.04	2.59E-03	6.13E-02	2',3'-cyclic nucleotide 3' phosphodiesterase
Lpcat4	99010	90	182.87	2.03	2.86E-03	6.45E-02	lysophosphatidylcholine acyltransferase 4
Lpin1	14245	36.14	73.26	2.03	4.26E-03	8.10E-02	lipin 1
Tcl1b4	27380	83.13	167.59	2.02	4.51E-03	8.31E-02	T cell leukemia/lymphoma 1B, 4
Pitpnc1	71795	65.1	131.57	2.02	2.95E-04	1.61E-02	phosphatidylinositol transfer protein, cytoplasmic 1
Meis1	17268	52.09	105.03	2.02	3.22E-04	1.72E-02	Meis homeobox 1
Gm839	330379	36.83	74.36	2.02	3.82E-03	7.67E-02	predicted gene 839
Pvrl1	58235	215.2	433.12	2.01	4.36E-03	8.18E-02	poliovirus receptor-related 1

APPENDIX A (continued)

Symbol	EntrezID	Mean of Intensities		Fold-change	p-value	FDR	Name
		WT	GATA4/6^{gcko}				
Tgfb1i1	21804	138.73	279.25	2.01	1.25E-03	3.99E-02	transforming growth factor beta 1 induced transcript 1
Tnfrsf1a	21937	121.54	243.9	2.01	6.89E-03	1.06E-01	tumor necrosis factor receptor superfamily, member 1a
Mboat1	218121	107.93	217.07	2.01	3.74E-04	1.89E-02	membrane bound O-acyltransferase domain containing 1
Dgka	13139	65.66	132.24	2.01	5.34E-03	9.16E-02	diacylglycerol kinase, alpha
Sntb1	20649	53.89	108.17	2.01	2.55E-05	3.45E-03	syntrophin, basic 1

TABLE V. GENES REGULATED IN THE ABSENCE OF BOTH GATA4 AND GATA6.

Genes are separated in downregulated and upregulated lists and organized in descending fold changes. The Symbol and EntrezID columns contain hyperlinks to the specific Gene page on the NCBI Entrez database.

APPENDIX A (continued)

TABLE VIA. GENES DOWNREGULATED IN ANIMALS WITH THE FOLLOWING GENOTYPES:

$G4^{gcko}$ (78)		$G6^{gcko}$ (14)	$G4/6^{gcko}$ (97)		
Cyp17a1	Luzp4	1700024J04Rik	Inhba	Calm1	Raver2
Cd34	Mir598	4933402N22Rik	Slc26a7	Abcd2	Hmgb2
Nup62cl	Myo18b	A430089I19Rik	Enc1	Pla2g4a	Dsel
Zpld1	Parm1	Akr1c18	Pip5k1b	BC057022	Zcchc16
1110032F04Rik	Vmn2r28	Cma2	Slfn4	Hsd17b1	Zdhhc2
Rgs13	Defa21	Gm13271	Trib2	Piga	Il1r1
Etohi1	Hunk	Gm5458	Dock4	Mfsd7c	Pgk1
St3gal1	Mup11	Gm5891	Ncrna00086	Asah2	Tnni3
Susd4	Olfr1034	Hist1h2ab	D830030K20Rik	Gm10406	
Lrrtm3	Olfr460	Mpp7	Nppc	Phex	
5031410I06Rik	Prickle2	Olfr1371	Gch1	Pparg	
Gm10220	Tmem178	Olfr1373	Inhbb	Myo6	
Rrs1	Vmn2r122	Olfr533	Pik3cg	Hist1h2br	
Slxl1	BC056474	Rplp0	Hey2	Prelid2	
Ces2g	Cdk18		Gpr126	Csrp2	
Chst15	Cebpa		Tulp2	Usp3	
Gm10471	Iqgap2		Adipor2	Speer8-ps1	
Rem1	Olfm1		Bub1	Gprc6a	
Amy2a5	Pdlim2		Jak1	Epha7	
Cacna1d	Rab11fip1		Coasy	Fbxl22	
Fn1	Armcx2		Atp10a	Tmem45a	
Mup2	Ctsh		Gm129	Cdkn2c	
Gm14354	Gm15107		Lhcgr	Rps6ka2	
Pcx	Gm5622		Rasgrp4	Ddah1	
1700084J12Rik	Gpc6		Slc16a3	Slc38a5	
D14Ertd449e	Lsr		Idi1	Ephx2	
Prrg1	Mfge8		Lzts1	Ldlr	
Agrn	Prlr		Erdr1	Etl4	
Enpep	Tuba1a		Chst5	Fam13a	
Gm5589	Avpi1		St3gal4	Cnot6	
Gm6682	Fam196a		Mir503	Rnd2	
Mlh1	Scoc		Jam2	Ralb	
Mup7	Vmn2r85		Wapal	Nckap5	
Npr1			Srbd1	Hist1h2bb	
Olfr1383			Tes	Hmgcr	
Ott			Prkar2b	Neb	
Robo2			Hipk2	Mfsd2a	
Vmn2r34			Ces1b	Rnf128	
Adamts12			Gdnf	Scgb3a1	
Il28a			Rassf4	Gcnt4	
Abpz			Map3k5	Lrrc2	
D0H4S114			Klc2	Sdf2l1	
Ndn			Bex4	Greb1l	
Aplnr			Ext1	Bnip3	
Bves			Spin2		

APPENDIX A (continued)

TABLE VIB. GENES DOWNREGULATED IN COMMON BETWEEN THE FOLLOWING GENOTYPES:

$G4^{gcko}$ and $G4/6^{gcko}$ (51)		**$G4^{gcko}$ and $G6^{gcko}$ (11)**	**$G6^{gcko}$ and $G4/6^{gcko}$ (4)**	**$G4^{gcko}$ and $G6^{gcko}$ and $G4/6^{gcko}$ (5)**
Gabrb2	**Cyp19a1**	4933409K07Rik	Acp1	**Comp**
Ctsc	Satb2	Gm3893	Amy1	Adh1
Grem1	Chst1	Krtap16-4	**Grem2**	Gstm6
Tnfsf11	Alms1	Gm5168	Pdgfrl	Vmn2r43
Masp1	Ralgapa2	Igk		C130026I21Rik
Lypd6	Rhox8	Dad1		
Pappa	Aldh1a1	Vmn1r79		
Plp1	Mapre2	Gm5114		
Cdh2	Elovl2	Olfr1200		
Defb19	Ryr2	Vmn1r221		
Rimklb	Slc7a8	Vmn2r60		
Rassf2	Tmsb15l			
Gabra1	Ccdc68			
Mro	Dock5			
Gsta4	Hcn1			
Lect1	P2ry13			
Tom1l1	Xlr5a			
Arrdc4	Cyb5			
Mapkbp1	Vcan			
Fdx1	**Adamts1**			
Plxnc1	Igsf3			
Tmeff2	Fam162b			
Tox	Mid1ip1			
Fshr	Slc12a7			
Ccbl2	Snord7			
Sema3g				

APPENDIX A (continued)

TABLE VIC. GENES UPREGULATED IN ANIMALS WITH THE FOLLOWING GENOTYPES:

$G4^{gcko}$ (25)	$G4/6^{gcko}$ (282)						
Ano4	Dgka	Angptl4	Atp1b1	Slc19a3	Bcl2l10	Slc10a4	Ren1
Fam46a	Sntb1	Qtrt1	Rab11fip5	Lamb1	Itm2a	Lyz2	Gria3
Gca	Tgfb1i1	Sema3d	Lims2	Akr1b10	B4galt1	Sorl1	Ogn
Bche	Tnfrsf1a	Slc19a2	Smpdl3a	1Rik	S100a1	Adhfe1	Plxdc1
Synm	Pvrl1	Papln	Isyna1	Slc7a4	Sned1	Slc45a4	Gpx3
Tgfb3	Meis1	C87414	Cmbl	Dmrta1	Ctsf	Fbln2	Gm3579
Fam171b	Tcl1b4	Arg2	Lgals3bp	Cpxm1	Itgb8	Ace	Grik3
Fbn2	Pitpnc1	Tmem41a	Tmem231	Gpnmb	Oplah	Ptprz1	Tns4
Rhobtb1	Gm839	Setd7	Cited1	H2-D1	Gdpd5	Fbln7	Hsd3b6
Sord	Lpin1	Lphn2	Fbln5	Tcl1b1	Nrp2	Rgs9	Col12a1
Itga9	Lpcat4	Crip3	Tuba8	Slc43a2	Dkkl1	Pdgfra	Igfbp4
Rbms3	Cnp	Acot2	Ehd2	Sh3pxd2a	Klf9	Mamdc2	Penk
Myo1e	Pdpn	Oog1	Plat	Slc25a42	Apcdd1	Fstl3	
Plbd1	Msi1	Entpd1	Ptpre	Cfhr2	Zp2	Prkcb	
k	Mmp23	Eya2	Omd	Mrc2	Tmeff1	Spinlw1	
Cnnm1	Grasp	Rbl2	B4galt6	Cldn11	Zp3	Itih5	
Mycn	Uggt2	Slc25a23	Plekhb2	Deptor	Camsap3	Igfbp5	
Kazald1	Gpcpd1	Car12	Ednra	Hpse	Drp2	Gadd45g	
Kcnq5	Csdc2	Gdf9	Ltbp3	Rasgrp1	Pvt1	Bcat1	
Sel1l3	Fam126b	Glul	Tns1	Kcnk5	Hmga2	S100b	
Nfib	Mansc1	Slit3	Nup210	Mxra8	Rorc	Aspn	
Grin2c	H1foo	Kdelc2	C87977	Sgk3	Slc38a3	P4ha3	
Pik3ip1	9Rik	Daam2	Pknox2	Fabp3	Trpc4	Gxylt2	
Gjc3	Sik1	Gm97	Tfpi	Dbp	Tmem35	Gatm	
2010110P09Rik	Phxr4	Plin2	Smoc1	Fbp1	Steap2	Col8a1	
	Gm15698	Capg	Olfml2b	Abcc4	Ptch1	Gdpd3	
	Flrt1	Sema5b	Nobox	Gpr165	Nipal1	Htra3	
	Epha4	Wt1	Derl3	Padi6	Tspan4	Agt	
	Gas1	Ptges	Lbp	Gas6	Mgp	Doc2b	
	Arhgdig	Rtn4rl1	Abca1	Axl	Usp18	Mmd2	
	Mxra7	Gpr20	Itgb5	Selenbp1	Cacna1h	Coch	
	Acsf2	Cdh3	Rgs11	Ggt5	Gdpd2	Gmpr	
	Zfp36l1	Kcnh1	Slc24a3	Bcl6	Abcc3	Gm5294	
	Fmo1	Rab34	Chrd	Zp1	Gna14	Tmem171	
	Hes1	Slc7a5	Tmem176a	Adarb1	Gstt1	Pdcd1	
	Stc2	Tppp3	Abhd14a	Wfdc10	Lama2	Nrip2	
	Fbxw14	Cyp4f18	Crabp2	Ror1	Atp2b4	Enpp6	
	Sesn2	BC031353	Tbc1d9	Mosc2	Pcp4l1	Igdcc4	
	Kctd14	Slc4a11	Dpysl5	Map1lc3a	Ptgds	Lgi3	
	Hist1h2aa	Tk2	A4galt	Arhgap42	Fam198a	Apoe	
	Cfh	Rfpl4	Clec2d	Rnf19b	Phyhd1	Mmp2	
	Col6a1	Got2	Fcgrt	Ccno	Kcnt1	Srpx2	
	Pipox	Spint2	Fgf11	Pltp	Tnfrsf21	Amh	
	Sptlc2	H6pd	Nbl1	Uchl1	Spp1	Ptgis	
	Tmie	Cpa1	Sdk1	Wisp2	Lamc3	Tgfbi	

APPENDIX A (continued)

TABLE VID. GENES UPREGULATED IN COMMON BETWEEN THE FOLLOWING GENOTYPES:

$G4^{gcko}$and $G4/6^{gcko}$ (54)			
Cxx1c	Ssbp2	Gem	Eya4
Foxp2	AB041803	E330013P04Rik	**Pcsk6**
Sox18	Gyltl1b	Mboat1	Gadd45b
Mkx	Cd200	Gpr98	Nos2
Myc	Kcnip3	Ank2	Adck3
Fam78a	Angptl1	Emx2	Cwc22
Igfbp2	Polr3g	Rarres2	Slc18a2
Spon1	Gpm6b	Sytl2	Itih2
Mblac2	Klhl31	Gadd45a	Mapk10
Sorbs2	Adamts2	Fam110c	**Cyp1b1**
Maml2	Tgfbr3	Leprel1	Bcan
Sema4d	Kcnk1	Hpgd	Gm13691
Bpifb5	Sgpp2	Nupr1	
Nlrc5	Kcnma1	Limch1	

APPENDIX A (continued)

TABLE VII: FUNCTIONAL ENRICHMENT ANALYSIS (DAVID) OF DIFFERENTIALLY EXPRESSED GENES IN GATA4/6^{iGCKO} ANIMALS.

Gene Ontology (GO) Term	Count	%	P Value
GO:0043167~ion binding GO:0030955~potassium ion binding GO:0046872~metal ion binding GO:0005509~calcium ion binding	119	24.44	7.70E-03
GO:0005886~plasma membrane	108	22.18	1.95E-03
GO:0005576~extracellular region GO:0044421~extracellular region part GO:0050840~extracellular matrix binding GO:0005578~proteinaceous extracellular matrix GO:0005615~extracellular space	99	20.33	1.11E-12
GO:0007155~cell adhesion GO:0022610~biological adhesion	33	6.78	2.92E-05
GO:0015267~channel activity GO:0022803~passive transmembrane transporter activity GO:0022838~substrate specific channel activity	18	3.7	9.57E-03
GO:0008610~lipid biosynthetic process	17	3.49	3.44E-03
GO:0001944~vasculature development GO:0001568~blood vessel development GO:0001570~vasculogenesis	16	3.29	2.44E-03
fatty acid metabolic process GO:0006690~icosanoid metabolic process GO:0006692~prostanoid metabolic process GO:0006693~prostaglandin metabolic process	14	2.87	1.10E-03
GO:0008015~blood circulation GO:0003013~circulatory system process GO:0003018~vascular process in circulatory system GO:0050880~regulation of blood vessel size GO:0008217~regulation of blood pressure GO:0035150~regulation of tube size	12	2.46	1.52E-04
GO:0030247~polysaccharide binding GO:0001871~pattern binding GO:0005539~glycosaminoglycan binding GO:0008201~heparin binding	12	2.46	3.35E-04
GO:0042803~protein homodimerization activity	12	2.46	8.64E-03
GO:0043062~extracellular structure organization	11	2.26	5.76E-03
GO:0006813~potassium ion transport	11	2.26	9.35E-03
GO:0019838~growth factor binding GO:0005520~insulin-like growth factor binding	10	2.05	6.76E-05
GO:0046942~carboxylic acid transport GO:0015849~organic acid transport GO:0006865~amino acid transport GO:0015171~amino acid transmembrane transporter activity	10	2.05	1.12E-03
GO:0006869~lipid transport GO:0010876~lipid localization	10	2.05	3.98E-03
GO:0007548~sex differentiation	10	2.05	7.07E-03
GO:0042445~hormone metabolic process	9	1.85	2.41E-03
GO:0001666~response to hypoxia GO:0070482~response to oxygen levels	8	1.64	1.35E-03

APPENDIX A (continued)

Gene Ontology (GO) Term	Count	%	P Value
GO:0008081~phosphoric diester hydrolase activity GO:0008889~glycerophosphodiester phosphodiesterase activity	8	1.64	1.99E-03

TABLE VII. DAVID FUNCTIONAL ENRICHMENT OF DIFFERENTIALLY REGULATED GENES FOUND IN G4/6^{GCKO} ANIMALS.

Functions that were significantly enriched had a $P<0.01$. The number of genes listed denotes the number out of the total number significantly regulated in the individual knockouts. The percent is the number of genes found within that functional group divided by the total number of significantly regulated genes.

APPENDIX B

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

4/17/2013

Carlos Stocco
Physiology & Biophysics
M/C 901

Dear Dr. Stocco:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 4/17/2013.**

Title of Application: **Molecular Pathways Controlling Ovarian Gene Expression**
ACC NO: **12-057**
Original Protocol Approval: **5/22/2012 (3 year approval with annual continuation required).**
Current Approval Period: **4/17/2013 to 4/17/2014**

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 2

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Molecular Pathways Controlling Ovarian Gene Expression*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HD057110	*Funded*	*2010-00093*	*UIC*	*Carlos Stocco*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Regulation of Aromatase Expression in the Corpus Luteum*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R21 HD066233	*Funded*	*2010-05141*	*UIC*	*Carlos Stocco*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*kg*
cc: BRL, ACC File, Jill Bennett, Michael Bauschard, Yan Guang Wu

Phone (312) 996-1972 • Fax (312) 996-9088

APPENDIX C

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

August 13, 2012

Carlos Stocco
Physiology & Biophysics
M/C 901

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Stocco:

The protocol indicated below has been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago on 7/12/2012. *The protocol was not initiated until final clarifications were reviewed and approved on* ***08/01/2012. Protocol expires 3 years from the date of review (07/12/2015). This protocol replaces protocol 09-065 which has been terminated.***

Title of Application: Regulation of Gene Expression in Ovarian Cells

IBC Number: 12-042

Highest Biosafety Level: 2

Condition of Approval: ***The enclosed report indicates the training status for bloodborne pathogen (BBP) training. Only those personnel who have been trained and whose training has not expired are approved for work that may involve exposure to bloodborne pathogens. Please note that federal regulations require yearly training for BBP.***

You may forward this letter of acceptable IBC verification of your research protocol to the funding agency considering this proposal. **Please be advised that investigators must report significant changes in their research protocol to the IBC office via a letter addressed to the IBC chair prior to initiation of the change. If a protocol changes in such a manner as to require IBC approval, the change may not be initiated without IBC approval being granted.**

Thank you for complying with the UIC's Policies and Procedures.

Sincerely,

Randal C. Jaffe, Ph.D.
Chair, Institutional Biosafety Committee

RCJ/*ss*

Enclosures

Cc: IBC file, Jill Bennett, Ping Zhou

Phone (312) 996-1972 • Fax (312) 996-9088 • www.research.uic.edu

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VITA

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Bachelor of Science, Biology with minors in Organic Chemistry and Religious Studies.
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Research Experience

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Summer intern/volunteer and research assistant, 6/07-9/07; 1/08-8/08
Employed by Dr. Jeffrey Jensen and Dr. Mary Zelinski

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Accomplishments and Awards

Physiology and Biophysics Graduate Student Association president: May 2012-May 2013

Physiology and Biophysics Graduate Student Association vice president: May 2011-May 2012

Physiology and Biophysics Graduate Student Association secretary: May 2010-May 2012

Kate Barany Award. Department of Physiology and Biophysics: 2011

Graduate Student Council Travel Awardee. University of Illinois at Chicago: 2012

Larry Ewing Memorial Trainee Travel Fellow. Society for the Study of Reproduction: 2012 and 2013

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Society for the Study of Reproduction Trainee Research Poster Finalist: 2010 and 2013

Organizing committee member for Illinois Symposium on Reproductive Sciences in Health and Disease: 2010 and 2012

Coordinator/ organizer of Reproduction, Endocrinology and Development seminars at University of Illinois at Chicago: 2010-2013

Peer Reviewed Publications

Yan-Guang Wu*, **Jill Bennett***, Deepika Talla, and Carlos Stocco. Testosterone, not 5α-dihydrotestosterone, stimulates LRH-1 leading to FSH-independent expression of Cyp19 and P450scc in granulosa cells. *Molecular Endocrinology* 2011 25:656-68. * Equal contribution.

Jill Bennett, Yan-Guang Wu, Jan Gossen, Ping Zhou, and Carlos Stocco Loss of both GATA6 and GATA4 in Granulosa Cells Blocks Folliculogenesis, Ovulation and Follicle Stimulating Hormone Receptor Expression Leading to Female Infertility. *Endocrinology* 2012 May:153(5):2474-85.

Ping Zhou, Sarah J. Baumgarten, Yanguang Wu, **Jill Bennett**, Nicola Winston, Jennifer Hirshfeld-Cytron and Carlos Stocco. IGF-1 Signaling is Essential for FSH stimulation of AKT and Steroidogenic Genes in Granulosa Cells. *Molecular Endocrinology* 2013 March:27(3): 511-23.

Jill Bennett, Sarah Baumgarten, and Carlos Stocco. GATA4 and GATA6 Silencing in Ovarian Granulosa Cells Affects Levels of Messanger RNAs Involved in Steroidogenesis, Extracellular Structure Organization, IGF1 Activity and Apoptosis. *Accepted pending revisions, Encodrinology 2013*.

Abstracts/Presentations

Jill Bennett, Cassandra Richards, Kimberley Mullen and Carlos Stocco. Androgens Stimulate Aromatase and P450scc in Primary Rat Granulosa Cells Independently of Follicle Stimulating Hormone (2010). 43rd Annual Meeting for the Society for the Study of Reproduction. Milwaukee, WI. *Poster Presentation*

Jill Bennett and Carlos Stocco. GATA4 Role in Ovarian Granulosa Cell Function and Female Fertility (2010). UIC College of Medicine Research Forum. Chicago, IL. *Poster Presentation*

Jill Bennett and Carlos Stocco. GATA4 role in ovarian granulosa cell function and fertility (2010). 2nd Illinois Symposium on Reproductive Sciences in Health and Disease. Chicago, IL. *Poster Presentation*

Yan-Guang Wu, **Jill Bennett**, Deepika Talla and Carlos Stocco. Testosterone, not 5α-Dihydrotestosterone Stimulates LRH-1 Leading to FSH independent Expression of Cyp19 and P450scc in Granulosa Cells (2010). 2nd Illinois Symposium on Reproductive Sciences in Health and Disease. Chicago, IL. *Poster Presentation*

Jill Bennett. The Role of GATA4 and GATA6 in Granulosa Cell Function (2011). UIC Physiology and Biophysics Departmental Student Seminar. Chicago, IL. *Oral presentation.*

Jill Bennett and Carlos Stocco. The Role of GATA4 and GATA6 in Granulosa Cell Function (2011). 44th Annual Meeting for the Society for the Study of Reproduction. Portland, OR. *Oral Presentation*

Ping Zhou, Michael Bauschard, Yan-Guang Wu, **Jill Bennett** and Carlos Stocco. Mechanisms of IGF-1 and insulin Synergizing with FSH in Inducing Aromatase Expression in Granulosa cells (2011). 44th Annual Meeting for the Society for the Study of Reproduction. Portland, OR. *Poster Presentation*

Jill Bennett, Yan-Guang Wu, Ping Zhou and Carlos Stocco. Ovarian Granulosa Cell Expression of GATA4 and GATA6 Is Required for Female Mice Fertility (2011). 3rd Annual Illinois Symposium on Reproductive Sciences in Health and Disease. Champaign, IL. *Poster Presentation*

Jill Bennett, Yan-Guang Wu, Ping Zhou and Carlos Stocco. GATA Factors are Essential for Normal Ovarian Function, Female Fertility, and Follicle Stimulating Hormone Receptor Expression in Granulosa Cells (2011). UIC College of Medicine Research Forum. Chicago, IL. *Poster Presentation*

Jill Bennett. GATA4 and GATA6 are Necessary for Folliculogenesis, Fertility, Ovulation and Ovarian Gene Regulation (2012). UIC Department of Physiology and Biophysics Mid-thesis Defense. Chicago, IL. *Oral Presentation*

Jill Bennett and Carlos Stocco. GATA4 Silencing Affects Apoptosis, Steroidogenesis and IGF-1 Signaling Pathways in Ovarian Granulosa Cells (2012). 45th Annual Meeting for the Society for the Study of Reproduction. State College, PA. *Poster Presentation*

Jill Bennett, Yan-Guang Wu, Ping Zhou and Carlos Stocco. The Roles of GATA4 and GATA6 in Folliculogenesis, Fertility, Ovulation and Gene Regulation (2012). NIH Annual National Graduate Student Research Conference. Bethesda, MD. *Poster Presentation*

Jill Bennett and Carlos Stocco. GATA4 silencing affects steroidogenesis, extracellular matrix organization and IGF-1 signaling pathways in granulosa cells (2012). 4th Annual Illinois Symposium on Reproductive Sciences in Health and Disease. Chicago, IL. *Poster Presentation*

Jill Bennett, John Lyndon, Francesco DeMayo, and Carlos Stocco. Conditional deletion of GATA4 and GATA6 impairs progesterone synthesis and leads to female infertility (2013). 46th Annual Meeting for the Societ for the Study of Reproduction. Montreal, Quebec. *Poster Presentation*

Ping Zhou, **Jill Bennett** and Carlos Stocco. Inhibition of glycogen sythetase kinase 3β activity by insulin-like growth factor 1 potentiates follicle stimulating hormone-induced phosphorylation of GATA4 (2013). 46th Annual Meeting for the Society for the Study of Reproduction. Montreal, Quebec. *Poster Presentation*